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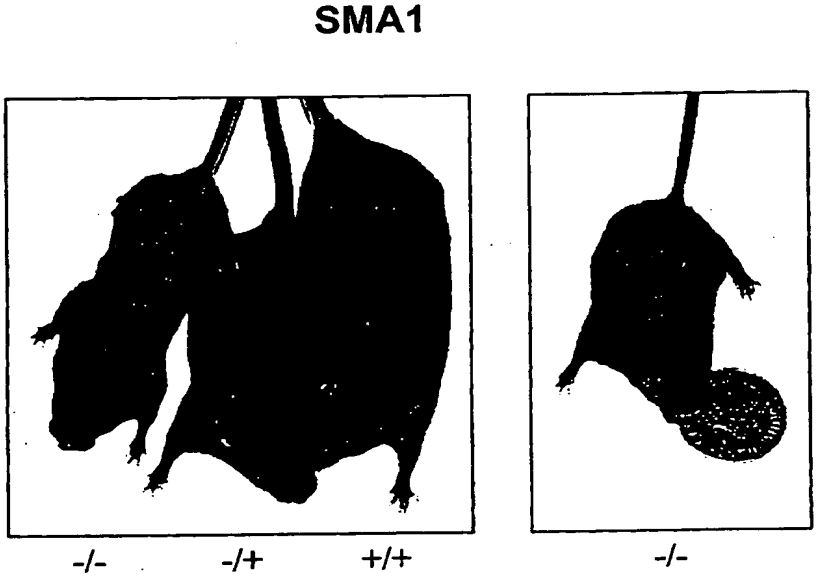
(54) Title: NON-HUMAN ANIMAL MODEL FOR GROWTH DEFICIENCY AND INFORMATION PROCESSING OR COGNITIVE FUNCTION DEFECTS AND USE THEREOF



(57) Abstract: The present invention provides a non-human animal model, particularly a mouse model, for growth deficiency and information procesing or cognitive function defects, in which modified growth hormone is expressed. The modified growth hormone and nucleic acids coding therefor are also provided, as are the correspondingly modified recombinant mouse and human growth hormones. The invention further provides uses for the non-human animal model and the modified growth hormones, in particular for the modified human growth hormone in treating medical conditions associated with over-expression of growth hormone or IGF-1.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Non-human animal model for growth deficiency and information processing or cognitive function defects and use thereof

Field of the Invention

The present invention relates to a non-human animal model, and particularly to a mouse model for growth deficiency and information processing or cognitive function defects. Furthermore, the present invention relates to a modified growth hormone, recombinant mouse and human growth hormones exhibiting the mutation and nucleic acid sequences coding therefor. The invention also relates to the uses of the modified growth hormones and nucleic acids for manufacturing medicaments for the treatment of diseases associated with over-expression or over-actvity of growth hormone or IGF-1.

Background of the Invention

Growth deficiency disorders

Proportionate short stature, accompanied by delayed growth velocity, and delayed skeletal maturation, is the most important clinical finding to support the diagnosis of growth hormone deficiency (GHD). While all these symptoms or signs are associated with GHD, none of them is specific for GHD. Additional findings of delayed bone maturation, absence of bone dysplasias, increased fat mass, decreased lean mass, decrease of intra and extra-cellular fluids, increased total cholesterol and low density lipoprotein (LDL), plus increased incidence of chronic disease are additional criteria (De Boer *et al.*, 1995; Endocr. Rev., 16: 63-86). Adequate function of the growth hormone (GH) pathway is needed throughout childhood to maintain normal growth.

Phillips and Cogan (1994; J. Clin. Endocr. Metab., 78: 11-16) reported four forms of isolated growth hormone deficiency (IGHD). IGHD IA and IB are both inherited in an autosomal recessive manner. In IGHD IA, deletions, frameshifts, and nonsense mutations lead to absent

GH with severe dwarfism; patients often develop anti-GH antibodies when given exogenous growth hormone. In IGHD IB, splice site mutations are responsible for low but detectable levels of GH. Dwarfism is less severe than in IGHD IA and patients usually respond well to exogenous GH. IGHD II has an autosomal dominant mode of inheritance and is caused by splice site or missense mutations that have dominant-negative effects. The clinical severity of IGHD II is variable between kindreds. Usually these patients also respond well to exogenous GH. IGHD III is an X-linked disorder that is often associated with hypogammaglobulinemia, suggesting a contiguous gene syndrome.

The occurrence of concomitant or combined deficiencies of other pituitary hormones (luteinizing hormone (LH); follicle-stimulating hormone (FSH); thyroid-stimulating hormone (TSH); and/or ACTH) in addition to GH is termed combined pituitary hormone deficiency (CPHD) or panhypopituitary dwarfism. The combination of GH and these additional hormone deficiencies often causes more severe retardation of growth and skeletal maturation and spontaneous puberty may not occur (Phillips, 1995; in Scriver *et al.* (eds) "*The metabolic and Molecular Bases of Inherited Disease*" vol. II (7th ed.) pub. McGraw-Hill, New York: 3023-3044). A significant reduction of bone mineral density (BMD) associated with abnormalities of bone turnover parameters was found only in patients with very severe or severe growth hormone deficiency (GHD), whereas normal BMD values were found in non-GHD hypopituitary patients.

Cases of growth hormone absence or deficiency, including both Illig-type deficiency (Cogan *et al.*, 1993; J. Endocrinol. Metab., 76: 1224-1228) and Kowarsky syndrome (Kowarsky *et al.*, 1978; J. Clin. Endocr. Metab., 47: 461-464. Takahashi *et al.*, 1996; New Eng. J. Med., 334: 432-436, 1207), have been attributed to mutations of the human growth hormone gene GH, which maps to Chr 17q (George *et al.*, 1981; Hum. Genet., 57: 138-141).

The treatment of GH deficiency is replacement using exogenous, biosynthetic GH. Factors important in the clinical response include the etiology and severity of deficiency, age of onset, and duration of replacement, as well as the sex of the affected individual. Burman *et al.* (1997; *J. Clin. Endocr. Metab.*, 82: 550-555) reported that men and women with GHD display marked differences in their responsiveness to GH replacement therapy. They suggested that these differences be taken into consideration when optimizing the treatment of GHD patients.

Acromegaly and gigantism are two clinical disorders resulting from an abnormally maintained activation of the GH-IGF-1 axis, especially with abnormally high plasma levels of GH. Symptoms of acromegaly include coarsening of facial features, excessive growth of hands and feet, soft tissue hypertrophy, increased perspiration, skin tags and tongue swelling. In addition excess GH in these patients is responsible for proliferation of articular cartilage which may undergo necrosis and erosion and endoneuronal fibrous proliferation which causes peripheral neuropathies. Excess GH also increases tubular reabsorption of phosphate and leads to hyperphosphatemia. Many of these symptoms are also seen in patients with gigantism. The role of IGF-1 in GH-mediated disorders such as acromegaly and gigantism are well recognized (Melmed *et al.*, 1994; J. Med., 97: 468-473).

Another disorder associated with abnormal GH levels is diabetes mellitus (DM). Patients with poorly controlled DM have been found to have high levels of circulating GH, hypophysectomy reducing the diabetic hyperglycemia (Houssay and Biasotti, 1930; Rev. Soc. Argent. Biol., 6: 251-296). It has been suggested that hypersecretion of GH may be the cause of poor diabetic control (Press *et al.*, 1984; New Engl. J. Med., 310: 810-814). The pathogenesis of diabetic retinopathy is believed to be mediated by GH (Rymaszewski *et al.*, 1991; Proc. Natl. Acad. Sci. USA 88: 617-621). Patients with long-standing diabetes will commonly suffer from diabetic retinopathy, one of the leading causes of blindness in the USA (Benson *et al.*, *Diabetic Retinopathy*, Duame (ed), pub. Harper and Row, Philadelphia PA, 1-24). Long term diabetic patients also exhibit angiopathy, peripheral neuropathy, and diabetic nephropathy evolving into glomerulosclerosis. Transgenic mice which overexpress bovine GH have been shown to have enlarged glomeruli which progressed to a state of glomerulosclerosis, implicating GH in the development of this nephropathology (Doi et al., Am. J. Pathol. 137: 41, 1990). The implication of high levels of GH in these pathologies is further supported by the fact that dwarfs with diabetes do not develop these type of proliferative pathologies seen in normal-sized diabetics (Merimee et al., 1978; New Engl. J. Med., 298: 1217-1222).

The present invention relates to proteins which are substantially homologous with growth hormone from various mammalian sources, but which have growth hormone-inhibitory activity. Growth hormone (GH) is a protein synthesized and secreted by acidophilic or somatotropic cells of the anterior pituitary gland (adenohypophysis). The regulation of GH synthesis and release is modulated by a family of genes that include the transcription factors PROP1 and PIT1. PROP1

and PIT1 regulate the differentiation of pituitary cells into somatotrophs which synthesize and release GH. Genes that are important in the release of GH include the growth hormone releasing hormone GHRH and its receptor GHRHR, whereas somatostatin inhibits GH release.

Human and mouse growth hormone have a molecular mass of 22,000 and contain 191 and 193 amino acid residues, respectively, with two conserved disulfide bridges (Niall *et al.*, 1971; Proc. Nat. Acad. Sci. USA, 68: 866-869), and four α-helix domains. GH has direct effects facilitating muscle growth and sparing glucose by increasing peripheral resistance to insulin; its skeletal growth effects are partly direct effects on chondrocyte differentiation and partly indirect effects via insulin-like growth factors on chondrocyte and muscle proliferation (Barton *et al.*, 1989; Cytogenet. Cell Genet., 50: 137-141). Moreover, experience in children and studies of acromegalic adults have demonstrated that GH has anabolic, lipolytic and antinatiuretic properties.

After its release into the blood, growth hormone binds its membrane-anchored receptor (GHR) as described by Goffin & Kelly (1996; Clinical Endocrinol., 45: 247-255). The X-ray crystal structure of human GH bound to its receptor revealed an asymmetric binding of one molecule of GH through two spatially distinct sites to two molecules of the GHR (Cunningham *et al.*, 1991; Science 254: 821-825). The dimerization of GHR that is induced by GH binding results in receptor activation and, among other effects, the induction of the expression of the insulin-like growth factor IGF-1. IGF-1 and its receptor (IGF1R) stimulate growth in various tissues including bones and muscle (Phillips, 1995; in Scriver *et al.* (eds) "*The metabolic and Molecular Bases of Inherited Disease*" vol. II (7th ed.) pub. McGraw-Hill, New York: 3023-3044. Rimoin and Phillips, 1997; in Rimoin *et al.* (eds.) "*Principles and Practice of Medical Genetics*" vol. I (3d ed.) pub. Churchill Livingstone, New York: 1331-1364.) and the encoding nucleic acid sequences belong to the major genes involved in the GH transduction pathway. GH molecules that are bound to membrane-anchored GH receptors can be released into the circulation by excision of the extracellular portion of the GHR molecules. At this point, the extracellular portion of the GHR, which is referred to as the GHBP, serves to stabilize GH in the circulation.

Smith *et al.* (1997; Science, 276: 1706-1709) demonstrated a role of GH in retinal neovascularization, which is the major cause of untreatable blindness. They found that retinal neovascularization was inhibited in transgenic mice expressing a GH antagonist gene and in

normal mice given an inhibitor of GH secretion. In these mice retinal neo-vascularization was inhibited in inverse proportion to serum levels of GH and IGF-1. Inhibition was reversed with exogenous IGF-1 administration. GH inhibition did not diminish hypoxia-stimulated retinal vascular endothelial growth factor (VEGF) or VEGF receptor (VEGFR) expression. Smith *et al.* (1997; Science, 276: 1706-1709) suggested that systemic inhibition of GH or IGF-1, or both, may have therapeutic potential in preventing some forms of retinopathy.

In the mouse GH expression begins at embryonic day 15.5, with dramatic increases in somatotroph numbers and signal intensities through day 17.5. Precursors for each of the various hormone producing cell types are committed early in pituitary development (Japon *et al.*, 1994; J. Histochem. Cytochem., 42: 1117-1125).

Morgan *et al.* (1987; Science, 237: 1476-1479) showed that retrovirus-mediated gene transfer can be used to introduce a recombinant human GH1 gene into cultured human keratinocytes. The transduced keratinocytes secreted biologically active GH into the culture medium. When grafted as an epithelial sheet onto athymic mice, these cultured keratinocytes reconstituted a normal-appearing epidermis from which, however, human growth hormone could be extracted. Transduced epidermal cells may be a general vehicle for the delivery of gene products by means of grafting.

The advent of transgenic technology provided the methods for production of pharmaceuticals by isolation of these proteins from the blood of transgenic animals. The mammary gland has been focused on as a bio-reactor since milk is easily collected from lactating animals and protein production can reach as high as 1 kg per day in cattle and 200 g per day in goats. Mammary-specific promoters have been used in transgenic animals to limit transgene expression to the mammary gland. Archer *et al.* (1994; Proc. Nat. Acad. Sci. USA, 91: 137-141) used gene therapy techniques to target a foreign gene to a single organ. They directly infused replication-defective retroviruses encoding the human growth hormone gene into the mammary gland of goats via the teat canal during a period of hormone-induced mammogenesis. This resulted in the secretion of human GH into the milk when lactation commenced on day 14 of the regime.

In mice homozygous for the 'little' mutation of the growth hormone releasing hormone receptor gene, $Ghrhr^{lit}$, somatotrophs lack secretory granules, while Ames dwarf ($Prop1^{df}$) and

Snell dwarf ($Pit1^{dw}$) homozygotes entirely lack somatotrophs. GH transcripts are also lacking from the latter two dwarf types, while little mutant mice showed a deficiency of such transcripts (Cheng *et al.*, 1983; Endocrinology, 113: 1669-1678). Ambiguous cells resembling either somatotrophs or mammotrophs (prolactin secreting cells) in pituitaries of Snell dwarf mice contain only adrenocroticotropic hormone granules (Wilson *et al.*, 1993; Anat. Rec., 236: 671-678).

Mice carrying the bovine GH gene transgenically express the hormone in heart and striated muscle at 5 to 6 weeks of age, with enhanced growth from six weeks old. There are no pathological effects on myocardium or striated muscle fibers, but older animals have severe glomerulosclerosis and altered liver metabolism (Conti *et al.*, 1995; Growth Regul. 5: 101-108). Transgenic normal and dwarf mutant bovine growth hormone have different effects in high-growth selected and unselected mouse lines. Normal bovine GH increases growth in the unselected line more than in the selected, though there is still a significant increase in the selected line. The dwarf mutant bovine GH decreases growth in selected mice more than in the unselected line, and also decreases or eliminates the greater growth observed in male than in female progeny (Eisen *et al.*, 1993; Theor. Appl. Genet., 87: 161-169).

Allelic differences identified by restriction fragment length variation occur among inbred strains of mice (Elliot *et al.*, 1990; Genomics, 8: 591-594). Gh maps to Chr 11 in the mouse using somatic cell hybrids (Jackson-Grusby *et al.*, 1988; Endocrinology, 122: 2462-2466) Intraspecific and intersubspecific backcrosses confirm the location of Gh on distal Chr 11 (Elliot *et al.*, 1990, Genomics, 8: 591-594), although genes for other hormones of this family are located on Chr 13 (Jackson-Grusby *et al.*, 1988; Endocrinology, 122: 2462-2466).

There is no animal model with an endogenous growth hormone mutation able to reflect human diseases associated with growth hormone deficiency. Without such an animal model the causes and precise physiological effects of such diseases are difficult to determine, the development of potential methods of treament is inhibited and comparable studies can only be implemented with considerable caution. Hence there is an unfulfilled need for an animal model suitable for laboratory study of the defects associated with bioinactivity of growth hormone or the results of antagonistic activity.

Similarly, there is a need for growth hormone antagonists and inhibitors to counter the many serious effects of over-expression or over-activity of growth hormone, IGF-1 and related disorders, as detailed above. Furthermore, there is a need for such antagonists and inhibitors to counter the undesirable secondary effects of certain drugs known in the art to aggravate or mimic such conditions when used for distinct therapeutic applications. There is a particular need for growth hormone antagonists and inhibitors which are immunologically compatible with treatment of human patients.

Summary of the Invention

The technical problems described above are addressed and solved by the subject matter of the present invention as described below. The present invention provides a non-human animal useful as a model of growth retardation, GHD related defects and information processing (cognitive function) deficiencies. The animal of the invention carries a mutated growth hormone gene encoding a growth hormone with a modified amino acid sequence compared to the wild type sequence.

The present invention also relates to the use of the animal model of the invention for the study of disorders associated with growth hormone activity deficiency, preferably information processing (cognitive function) defects and especially growth retardation and deficiency; as well as for the dissection of the growth hormone mechanism of action pathway, especially the identification of related or downstream genes and proteins involved in information processing and cellular growth.

In a further aspect the present invention provides a method for screening of preventive or therapeutic agents of disorders and symptoms associated to growth hormone activity deficiency, growth retardation or deficiency, dwarfism, Illig-type deficiency, Kowarsky syndrome, information processing- or cognitive function-related defects, age-related memory and behaviour deficits, osteoporosis and cardiovascular disorders, disorders of glucose metabolism, and increased body fat accumulation, by using the animal model of the invention.

In a still further aspect the present invention provides cell lines derived from the animal model of the present invention.

Furthermore, the present invention provides growth hormones having a modified amino acid sequence compared to the wildtype sequence and nucleic acids coding therefor. The association of an identified biochemical defect with the animal model of the present invention provides as an additional aspect of the present invention, isolated growth hormone corresponding to that produced by the non-human animal model of the invention, particularly mutant mouse growth hormone protein having a point mutation at position 193 (see SEQ ID NO:3). Recombinantly generated GH proteins providing the equivalent point mutation and variants with alternative amino acid substitutions are also provided, particularly mouse and human recombinant mutant and variant GH proteins. Another aspect of the invention provides the nucleic acid sequences encoding these sequences, the vectors and cell lines required to express the recombinant GH proteins, antibodies binding to these modified growth hormone proteins and chimeric protein derivatives of the growth hormone variants.

Further aspects of the invention provide the growth hormones of the present invention in which said growth hormones act as antagonists of endogenous GH activity; *i.e.* they exhibit growth hormone inhibitory activity; in particular where the growth hormones of the present invention act in a dominant negative manner. Consequently compositions comprising the growth hormone proteins of the present invention plus a pharmaceutically acceptable carrier are provided. Additional aspects provide the growth hormone proteins of the present invention, the polynucleotide encoding them and vectors bearing said polynucleotides for the prevention, treatment or amelioration of a medical condition in a mammalian subject, particularly a human subject, and in particular their use for the manufacture of a medicament for prevention, treatment or amelioration of any of the following medical conditions:

(i) excessive or undesirable growth rate;
(ii) over-activity or undesirable activity of endogenous growth hormone;
(iii) over-expression, over-production or undesirable production of endogenous growth hormone;
(iv) an excessive or undesirable condition shown to be modulated by endogenous growth hormone;
(v) over-activity or undesirable activity of endogenous IGF-1;
(vi) over-expression, over-production or undesirable production of endogenous IGF-1;

(vii) an excessive or undesirable condition shown to be modulated by endogenous IGF-1 activity;

(viii) excessive or undesirable proliferation of microvascular cells, wherein the proliferation is stimulated by endogenous growth hormone;

(viii) tumorogenesis, wherein the tumour's growth is stimulated by endogenous growth hormone;

(ix) excessive or undesirable serum concentration of cholesterol or low density lipoprotein (LDL);

(x) peripheral neuropathy;

(xi) glomerulosclerosis;

(xii) diabetes;

(xiii) acromegaly, gigantism and associated disorders;

(xiv) excessively lean body mass or a deficiency in body fat content.

Methods of diagnosis of the expression of the modified growth hormone of the invention are an additional aspect of the invention.

Brief Description of the Figures

- **Fig. 1** presents a picture of the SMA1 animals compared to wildtype mice demonstrating the reduced proportionate stature of the mutant animal. +/+, wild type C3HeB/FeJ mouse; -/+ heterozygous and -/- homozygous C3HeB/FeJ-SMA1 mutant mice.

- **Fig. 2** presents a growth curve of the descendants of C3HeB/FeJ-SMA1 animals compared to C3HeB/FeJ-wild type animals. Clearly, half of the (C3HeB/FeJ-SMA1$^{+/-}$ x C3HeB/FeJ-wild type) progeny displays a reduced growth curve and a reduced weight demonstrating the presence of a dominant inherited trait according to the Mendelian rules of genetic distribution, with a 100 % penetrance.

- **Fig. 3** presents the length of certain body parts of the (C3HeB/FeJ-SMA1 x C3HeB/FeJ-wild type) descendants compared to C3HeB/FeJ-wild type mice, and demonstrates that the C3HeB/FeJ-SMA1 heterozygous mutant mice have short but proportionate body parts.

Fig. 4 presents values for body mass and naso-anal length of wild type C3HeB/FeJ mice, C3HeB/FeJ-SMA1 heterozygous and C3HeB/FeJ-SMA1 homozygous mutant mice at eight weeks of age.

- **Fig. 5** presents a comparison of body fat to body weight for wild type C3HeB/FeJ mice and C3HeB/FeJ-SMA1 heterozygous mutant mice, demonstrating that the heterozygous animals exhibit a significantly higher body fat content (lower lean-to-fat ratio).

- **Fig. 6** presents the insulin-like growth factor-1 (IGF-1) plasma levels as measured by ELISA in C3HeB/FeJ-SMA1 heterozygous mutant and C3HeB/FeJ-SMA1 or C3HeB/FeJ-wild type mice. This demonstrates clearly that C3HeB/FeJ-SMA1 heterozygous mutant mice, have lower plasma levels of IGF-1 as compared to the C3HeB/FeJ wild type mice or C3HeB/FeJ-SMA1 x C3HeB/FeJ-wild type descendant mice without the phenotype.

- **Fig. 7** presents the growth hormone (GH) plasma levels as measured by ELISA in C3HeB/FeJ-SMA1 heterozygous mutant and C3HeB/FeJ-SMA1 or -wild type mice. This demonstrates clearly that C3HeB/FeJ-SMA1 heterozygous mutant mice have a subnormal to normal GH plasma levels as compared to C3HeB/FeJ wild type mice or C3HeB/FeJ-SMA1 x C3HeB/FeJ-wild type descendant mice without the phenotype.

- **Fig. 8** presents microscopic pictures of histological transversal section of the pituitary gland demonstrating the size reduction (A and B) of the anterior lobe of the adenohypophysis and the absence of secretion granules in the acidophilic cells of the adenohypophysis (C and D) characteristic of the C3HeB/FeJ-SMA1 heterozygous mutant animals compared to the C3HeB/FeJ wild type mouse.

- **Fig. 9** presents a 2-D gel electrophoretic analysis of the liver proteome from the C3HeB/FeJ-SMA1 homozygous mutant mouse compared to that from the C3HeB/FeJ wild type mouse.

- **Fig. 10** presents the identity of differentially expressed proteins in the liver of SMA1 C3HeB/FeJ-SMA1 homozygous mutant mouse compared to those expressed in the liver of C3HeB/FeJ wild type mice.

- **Fig. 11** presents the chromosome localization of the SMA1 associated mutation (A), the microsatellite analysis of the haplotype of [(C3HeB/FeJ- SMA1$^{+/-}$ x C57Bl/6Jico) x C57Bl/6Jico] mice (B) and the chromatograms resulting from the sequencing of a part of the 5th exon of the Gh gene in C3HeB/FeJ-SMA1 homozygous and heterozygous mutant animals and in C3HeB/FeJ wild type mice (C). This demonstrates that the SMA1 associated mutation locates on chromosome 11 (A), between the microsatellite markers D11Mit333 and D11Mit301 (B) and is a A (639) to C transition (codon 193, amino acid 193) located in the 5th exon of the growth hormone Gh gene.

- **Fig. 12** presents the difference in the *Ava*II restriction pattern between wild type and mutant nucleic acid sequence coding for growth hormone. This demonstrates the opportunity to detect the presence of the point mutation in the genomic nucleic acid sequences of heterozygous mutants C3HeB/FeJ-SMA1$^{+/-}$ and homozygous mutants C3HeB/FeJ-SMA1$^{-/-}$compared to wild type C3HeB/FeJ mice due to the disappearance of an AvaII restriction site in the nucleic sequence of the mutated growth hormone gene.

- **Fig. 13** presents the amino acid translation of the nucleic acid sequence of the SMA1 mouse mutant animals compared to the wild type of many other species demonstrating that the point mutation leads to a D to G amino acid transition, and is located in the fourth conserved α-helix domain at the C-terminus of GH.

- **Fig. 14** presents a schematic representation of a method for the construction and cloning of a recombinant DNA vector for point mutation at the position of the mouse growth hormone nucleic acid sequence.

- **Fig. 15** presents a representative SDS-PAGE analysis of the purification of recombinant mouse and human growth hormone (mGH and hGH, respectively), both wild type (wt) and SMA1 mutant (mut) variants, each expressed in *E. coli* M15 cells and purified from the bacterial inclusion bodies under denaturing conditions (as described in Example 9). The location at which GH migrated in the gel was confirmed by immunoblotting, and is indicated by an arrow.

- **Fig. 16** presents a representative SDS-PAGE analysis of the purification of recombinant mouse and human growth hormone (mGH and hGH, respectively), both wild type (wt) and

SMA1 mutant (mut) variants, each expressed in *E. coli* M15 cells and purified from the supernatant of the bacterial lysate under non-denaturing conditions (as described in Example 9). The location at which GH migrated in the gel was confirmed by immunoblotting, and is indicated by an arrow.

Detailed Description of the Invention

The present invention provides a non-human animal model which expresses a growth hormone protein modified compared to the amino acid sequence of the wild type protein. The growth hormone expressed may have similarity in sequence and secondary structure to a vertebrate growth hormone, including, but not limited to mammalian growth hormones, preferably bovine growth hormone and growth hormone from rat, and in particular growth hormone from mouse. The animal is preferably from a genus selected from the group consisting of *Mus* (*e.g.* mice), *Rattus* (*e.g.* rats), *Oryctologus* (*e.g.* rabbits) and *Mesocricetus* (*e.g.* hamsters). In a particularly preferred embodiment the animal is a mouse. Animals carrying a mutated growth hormone gene expressing said modified growth hormone exhibit one or more of the following phenotypical features:

- small proportionate stature characterized by reduced body weight and length, and reduced size of all body parts and organs;
- increased body fat content;
- non-proportionate reduced size and defective histology of the anterior pituitary gland;
- significant concentrations of circulating endogenous GH comprising the sequence of SEQ ID NO:3;
- levels of total GH in the plasma that are comparable, but significantly lower than in the wild type animals;
- abnormally low IGF-1 plasma levels;
- reduced O_2-consumption;
- normal body temperature;
- reduced or defective cognitive functions and information processing capacity.

The term "modified" according to the present invention refers to an alteration compared to the wild type. The term "phenotype" according to the invention refers to a collection of

morphological, physiological, behavioral and biochemical traits possessed by a cell or organism that results from the interaction of the genotype and the environment. Thus, the animal model of the present invention displays readily observable abnormalities. In a preferred embodiment the animal of the invention shows at least 2, preferably at least 4, more preferably 6 and most preferably all of the above listed phenotypical features.

Pursuant to the present invention mice were generated carrying a point mutation in the 5th exon of the mouse growth hormone gene as shown in SEQ ID NO:1 of the sequence listing, thereby replacing the aspartate residue at position 193 in the fourth helix domain at the C-terminus of the protein. Position 193 according to the present invention refers to the non-mature protein. However, it will be appreciated by the person skilled in in art that also the mature protein is encompassed by the present invention and may be expressed in the animal model of the present invention, which results from a cleavage of the first 26 amino acids. This modification of growth hormone results in the above mentioned phenotypical features. The aspartate residue at position 193 in the amino acid sequence of SEQ ID NO:1 and SEQ ID NO:2 is conserved among the growth hormones of different species as can be seen in the alignment of different members of the growth hormone family shown in Figure 13.

Thus, the non-human animal model of the present invention carries a nucleic acid sequence encoding growth hormone, whereby the codon for the amino acid at position 193 of the amino acids sequence shown in SEQ ID NO:1 and SEQ ID NO:2 or the codon corresponding to said position in other growth hormones, which encodes an aspartate in the wildtype, is mutated to encode an amino acid different from aspartate. In a preferred embodiment the animal model of the present invention expresses the amino acid sequence shown in SEQ ID NO:3. By "nucleic acid sequence" as used herein is meant any contiguous sequence series of nucleotide bases and may be ribonucleic acids and deoxyribonucleic acids. Preferably the nucleic acid sequence is cDNA. The expression "corresponding to said position in other growth hormones" refers to the amino acid position of growth hormones of various species matching the aspartate residue at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 in a homology alignment; see Figure 13.

In a preferred embodiment the animal model of the invention carries a modified growth hormone nucleic acid sequence derived from a vertebrate, preferably from a mammal, in

particular from mouse. In a particularly preferred embodiment the nucleic acid sequence is derived from the nucleic acid sequence shown in the sequence listing as SEQ ID NO:1.

Preferably, the aspartate residue of the modified growth hormone protein of the present invention is replaced by an amino acid with different size and/or polarity; i.e. a non-conservative amino acid substitution. Non-conservative substitutions are defined as exchanges of an amino acid by another amino acid listed in a different group of the five standard amino acid groups shown below:

1. small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. negatively charged residues and their amides: Asn, Asp, Glu, Gln;
3. positively charged residues: His, Arg, Lys;
4. large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys);
5. large aromatic residues: Phe, Tyr, Trp.

Conservative substitutions are defined as exchanges of an amino acid by another amino acid listed within the same group of the five standard amino acid groups shown above. Three residues are parenthesized because of their special role in protein architecture. Gly is the only residue without a sidechain and therefore imparts flexibility to the chain. Pro has an unusual geometry which tightly constrains the chain. Cys can participate in disulfide bonds.

Preferably the aspartate residue of growth hormone according to the present invention is replaced by an amino acid other than Asn, Glu, Gln and preferably by an amino acid selected from the group consisting of alanine, serine, threonine, proline and glycine, more preferably by proline, alanine and glycine and most preferably by glycine. In the most preferred embodiment the growth hormone expressed in the animal model of the present invention has the amino acid sequence shown in SEQ ID NO:4.

The animal model of the invention preferably expresses a modified growth hormone protein in all of its cells, and particularly in pituitary acidophilic cells, however, animals which express the modified growth hormone protein in some, but not all cells, which are termed cellular mosaic animals, are also comprised. The present invention further provides for inbred successive lines of animals carrying the mutant growth hormone nucleic acid of the present invention that offer the advantage of providing a virtually homogenous genetic background. A

genetically homogenous line of animals provides a functionally reproducible model system for disorders or symptoms associated with growth hormone activity deficiency, preferably information processing or cognitive function disorders and, particularly growth retardation or deficiency and associated disorders, as described above.

The present invention is not limited to the modification of the aspartate residue at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 or at a corresponding position in other growth hormones, which is necessary to impart the desired phenotype to the animal of the present invention. Rather it encompasses additional mutations in the growth hormone as long as they do not result in a loss of the phenotype. Such mutations include single or multiple further amino acid substitutions, deletions and insertions. Amino acid insertional derivatives of the present invention include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Preferably the growth hormone of the invention is at least 40 %, preferably at least 50 %, more preferably at least 80 % and most preferably at least 90 % homologous with the wildtype growth hormone sequence from vertebrate, preferably from mammals, most preferably from bovine, and from rat and particularly from mouse (SEQ ID NO:1). In a particularly preferred embodiment the polypeptide is identical with the wildtype sequence with the exception of the replacement of the aspartate residue at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 or at the corresponding position in other growth hormone sequences. Preferred modifications of the growth hormone amino acid sequence of the present invention, in addition to the modification at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 or at a corresponding position, are at positions which are not conserved among the vertebrate growth hormones. Preferably, such modifications replace an amino acid with one of similar size and polarity. In terms of the kinds of additional substitutions which may be made, one may look first to analyses of the frequencies of amino acid changes between homologous proteins of different organisms. Based upon such analyses, conservative amino acid substitutions are defined as exchanges within any one of the five standard amino acid groups set forth above.

It will be appreciated that the animal model of the invention may carry a mutated growth hormone nucleic acid according to the present invention derived from the same species or from a different species. Preferably, the mutated growth hormone nucleic acid of the present invention is homozygous in the animals of the present invention. Preferably, transcription of the mutated growth hormone-gene of the present invention is under the control of the promoter sequence controlling transcription of the endogenous wildtype sequence of the animal, although a different promoter may be used.

The animals of the invention can be produced by using any technique known to the person skilled in the art; including but not limited to micro-injection, electroporation, cell gun, cell fusion, micro-injection into embryos of teratocarcinoma stem cells or functionally equivalent embryonic stem cells. The animals of the present invention may be produced by the application of procedures which results in an animal with a genome that incorporates/integrates exogenous genetic material in such a manner as to modify or disrupt the function of the normal growth hormone. The procedure may involve obtaining genetic material, or a portion thereof, which encodes a growth hormone. The isolated native sequence is then genetically manipulated by the insertion of a mutation appropriate to replace the aspartate residue at position 193 of the amino acid sequence SEQ ID NO:2 (as is indicated in SEQ ID NO:3) or the corresponding residue of other growth hormones. The manipulated construct may then be inserted into embryonic stem cells, *e.g.* by electroporation. The cells subjected to said procedure are screened to find positive cells; i.e. cells which have integrated into their genome the desired construct encoding an altered growth hormone. The positive cells may be isolated, cloned (or expanded) and injected into blastocysts obtained from a host animal of the same species or not. For example, positive cells are injected into blastocysts from mice, the blastocysts are then transferred into a female host animal and allowed to grow to term, following which the offspring of the female are tested to determine which animals are transgenic; *i.e.* which animals have an inserted exogenous mutated DNA sequence. One method involves the introduction of the recombinant gene at the fertilized oocyte stage ensuring that the gene sequence will be present in all of the germ cells and somatic cells of the "founder" animal. 'Founder animal' as used herein means the animal into which the recombinant gene was introduced at the one cell mouse embryo stage.

The animals of the present invention present a phenotype whose characteristics are representative of many symptoms associated with human growth hormone deficiency associated disorders, therefore making the animal model of the present invention a particularly suitable model for the study of these diseases. In particular the animal model of the invention presents a phenotype characterized by a reduced weight, a short proportionate stature, a reduced size of all body parts and organs, and an increased body fat content, which all are phenotypical characteristics of human diseases or symptoms associated with growth hormone deficiency, in particular growth retardation and deficiency disorders, such as dwarfism, Laron Syndrome, Illig-type deficiency, and the Kowarsky syndrome.

Moreover, since the animals of the present invention have growth hormone plasma levels comparable to those of wild type animals they present symptoms similar to those related to human growth retardation or deficiency disorders characterized by plasma levels of growth hormone gene product that are normal or subnormal, associated with a deficiency of growth hormone activity or complete bioinactivity, such as the Illig-type deficiency and Kowarsky syndrome or with antagonistic activities, such as secondary effects of certain therapeutic drug treatments.

Reduced bone mineral density (BMD) has been reported in patients with isolated GHD and/or multiple pituitary hormone deficiencies. A significant reduction of BMD associated with abnormalities of bone turnover parameters was found only in patients with very severe or severe GHD, whereas normal BMD values were found in non-GHD hypopituitary patients (Colao *et al.*, 1999; J. Clin. Endocr. Metab., 84: 1919-1924). These abnormalities were consistently present in all patients with GHD regardless of the presence of additional hormone deficits, suggesting that GHD plays a central role in the development of osteopenia in hypopituitary patients (Tobiume *et al.*, 1997, J. Clin. Endocr. Metab., 82: 2056-2061). The animals of the present invention have a reduced anterior pituitary gland and thin cortical bones. Therefore, the animals of the invention can be used to study of diseases or symptoms associated with pituitary gland deficiency, and/or reduced BMD, and/or osteoporosis.

Furthermore, it has been demonstrated in humans that low IGF-1 plasma levels are correlated with cognitive function and information processing defects (van Dam *et al.*, 2000; Growth Horm. IGF Res., 10 Suppl. B: S69). Elderly people that were given IGF-1 showed improvement of information processing and cognitive function capacity, whereas those with

higher levels of IGF-1 in the serum performed better in cognitive function tests (Aleman, *et al.*, 1999; J. Clin. Endocrinol. Metab., 84: 471-475. Aleman, *et al.*, 2000; Neuropsychobiology, 41: 73-78). Such studies support a body of research suggesting that with aging a disruption occurs in the relation between IGF-1 and GH secretion. In rodents, experiments with rats indicate that chronic infusion of IGF-1 may ameliorate age-related behaviour deficits, including aiding working memory and reference memory (Markowska *et al.*, 1998; Neuroscience, 87: 559-569). Further studies have shown the neurobehavioural dysfunction associated with traumatic brain injury to be ameliorated by chronic post-trauma administration of IGF-1 (Saatman *et al.*, 1997; Exp. Neurol. 147: 418-427).

Moreover, elevated blood pressure and enhanced myocardial contractility was described in mice with severe IGF-1 deficiency (Lembo *et al.*, 1996, J. Clin. Invest., 98:2648-2655), while several lines of evidence in human and rats already suggested an important role of IGF-1 in the cardiovascular physiology (Copeland *et al.*, 1994; J. Clin. Endocrinol. Metab., 79: 230-232. Wahlander *et al.*, 1992; Hypertension, 19: 25-32. Duerr *et al.*, 1995; J. Clin. Invest., 95: 619-627. Cittadini *et al.*, 1996; Circulation, 93: 800-809. Pfeifer *et al.*, 1999; J. Clin. Endocr. Metab., 84: 453-457). Therefore, the animals of the present invention can also be used for the study of diseases or symptoms thereof or symptoms associated with low IGF-1 plasma levels such as growth retardation and deficiency, glucose metabolism disorders, cognitive function and information processing defects, and cardiovascular disorders. The animals are particularly useful as a model for the study of diseases or symptoms associated with growth hormone activity deficiency or IGF-1 deficiency such as growth retardation or deficiency, in particular dwarfism, Illig-type deficiency, Laron Syndrome and the Kowarsky syndrome, pituitary gland deficiency, reduced BMD, osteoporosis, accumulation of body fat resulting from defective storage or metabolism of fat, information processing and cognitive functions defects, glucose metabolism disorders and cardiovascular disorders. In particular some phenotypic characteristics of the animal model of the present invention, including, but not restricted to IGF-1 plasma levels, growth rate, cognitive function and information processing capacity, can be monitored over a long period and in various environments to evaluate the profile and progression of such diseases in a controlled and standardized context, which permit the evaluation of the influence of external environmental factors.

The animals of the present invention can also be used to identify early diagnostic markers for diseases associated with growth hormone activity deficiency or IGF-1 deficiency such as

growth retardation or deficiency, in particular dwarfism, Laron Syndrome, Illig-type deficiency, and the Kowarsky syndrome, pituitary gland deficiency, reduced BMD, osteoporosis, accumulation of body fat resulting from defective storage of metabolism of fat, information processing and cognitive functions defects, glucose metabolism disorders and cardiovascular disorders. Surrogate markers, including but not limited to ribonucleic acids or proteins, can be identified by performing procedures of proteomics or gene expression analysis known in the art. For example procedures of proteomics analysis include, but are not restricted to, ELISA, 2D-gel (as illustrated in Fig.9), protein microarrays or mass spectrophotometric analysis of any organ or tissue samples, such as blood samples, or derivatives thereof, preferably plasma, at different age or stage of GH activity deficiency associated disease development, or symptom thereof. As a further example, gene expression analysis procedures include, but are not restricted to, differential display, cDNA microarrays, analysis of quality and quantity of ribonucleic acids species from any organ or tissue samples, such as blood samples, or derivatives thereof, at different age or stage of development of GH activity deficiency associated disease, or symptom thereof.

Among other results, partial analysis of the liver proteome of SMA1 and wild type mice by 2-D electrophoresis permits identification of certain differentially expressed proteins (Fig. 10) that can be used as markers for growth hormone deficiency and related disorders. These proteins include, but are not restricted to glutathione S-transferase, myosin light chain 1, major urinary protein 1 and major urinary protein 2 (Fig. 10). This also indicates the broad physiological effects of growth hormone deficiency.

The animal model of the present invention can be used to monitor the activity of agents useful in the prevention or treatment of the above-mentioned diseases or symptoms, such as growth retardation or deficiency, in particular dwarfism, Laron Syndrome, Illig-type deficiency, and the Kowarsky syndrome, pituitary gland deficiency, reduced BMD, osteoporosis, information processing and cognitive functions defects, glucose metabolism disorders and cardiovascular disorders. The agent to be tested can be administered to an animal of the present invention and for example the growth rate, IGF-1 plasma level, the information processing capacity, and/or the cognitive functions of the animal of the present invention can be monitored. In a further embodiment the animals of the invention may be used to test therapeutics against any disorders or symptoms that have been shown to be associated with growth hormone activity deficiency or IGF-1 deficiency.

The animals of the present invention can also be used as test model system for materials, including but not restricted to chemicals and peptides, particularly medical drugs, suspected of promoting or aggravating the above described diseases associated with growth hormone activity deficiency. For example the material can be tested by exposing the animal of the present invention to different time, doses and/or combinations of such materials and by monitoring the effects on the phenotype of the animal of the present invention, including but not restricted to growth rates, IGF-1 blood levels, bone mineralization, information processing capacities and cognitive functions.

Furthermore, the animals of the present invention may be used for the dissection of the molecular mechanisms of the growth hormone activity pathway, that is for the identification of downstream genes or proteins thereof regulated by growth hormone activity and deregulated in growth hormone activity deficiency associated disorders, particularly those genes and proteins involved in cellular growth and cognitive functions or information processing. For example this can be done by performing differential proteomics analysis, using techniques including but not restricted to 2D gel analysis, protein chip microarrays or mass spectrophotometry, on liver, adipose or brain tissues of the animal of the present invention, such as liver, adipose and brain cells which all express the growth hormone receptor (GHR) and are responding to GH stimuli.

The animal model of the present invention can be used to identify and clone so called modifier genes which are able to modify, aggravate, reduce or inhibit the phenotype associated with a growth hormone activity deficiency. Particularly, for this purpose, the animal model of the present invention can be mate to mice of different strains carrying a different genetic background, which gives the possibility to map the genes modifying the phenotype. For example, the animal model of the present invention, when produced in an C3HeB/Fe inbred strain background can be bred to C57Bl/6Jico inbred mice. The hybrid animals of this progeny, are then further bred, either in back-cross strategy with C57Bl/6Jico inbred mice again, or in an intercross strategy between each other. The modifier gene can be then mapped and cloned by using microsattelites or a single nucleotide polymorphism (SNP) strategy on the mice resulting from the backcross or intercross breeding that have been grouped with respect to their phenotype intensity.

The animals of the invention can also be used as a source of primary cells, including all cell types, and particularly the acidophilic cells of the pituitary glands, for cell culture experiment, including but not restricted to, the production of immortalized cell lines by any methods known in the art, such as retroviral transformation. Cells from the animals may advantageously exhibit desirable properties of both normal and transformed cultured cells; *i.e.* they will be normal or nearly normal morphologically and physiologically, but can be cultured for long, and perhaps indefinite periods of time. The present invention provides such primary cells and cell lines derived therefrom, obtained from the animals of the present invention. These primary cells or cell lines derived thereof may be used for the construction of an animal model of the present invention.

In other embodiments cell lines may be prepared by the insertion of a nucleic acid construct comprising the nucleic acid sequence of the invention or a fragment thereof comprising the codon imparting the above described phenotype to the animal model of the invention (*vide infra*). Suitable cells for the insertion include primary cells harvested from an animal as well as cells which are members of an immortalized cell line. Recombinant nucleic acid constructs of the invention, described below, may be introduced into the cells by any method known in the art, including but not limited to, transfection, retroviral infection, micro-injection, electroporation, transduction or DEAE-dextran. Cells which express the recombinant construct may be identified by, for example, using a second recombinant nucleic acid construct comprising a reporter gene which is used to produce selective expression. Cells which express the nucleic acid sequence of the invention or a fragment thereof may be identified indirectly by the detection of reporter gene expression.

The present invention also provides a modified growth hormone amino acid sequence whereby the wild type aspartate residue at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 or at the corresponding position in other growth hormone sequences is replaced by another amino acid residue and nucleic acid sequences coding therefor. Preferably the modified growth hormone has the amino acid sequence shown in SEQ ID NO:3. Preferably the growth hormone is derived from a mammal, in particular from mouse. In a particularly preferred embodiment the amino acid sequence is derived from the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2.

Preferably, the aspartate residue of the modified growth hormone protein is replaced by an amino acid with different size and/or polarity; i.e. a non-conservative amino acid substitution as defined above.

Preferably the aspartate residue of growth hormone according to the present invention is replaced by an amino acid other than Asn, Glu, Gln and preferably by an amino acid selected from the group consisting of alanine, serine, threonine, proline and glycine, more preferably by proline, alanine and glycine and most preferably by glycine. In the most preferred embodiment the growth hormone of the present invention has the amino acid sequence shown in SEQ ID NO:4.

The present invention is not limited to the mutation of the aspartate residue at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 or at a corresponding position in other growth hormones. Rather it encompasses additional modifications in the growth hormone. Such mutations include single or multiple further amino acid substitutions, deletions and insertions as defined above. Preferably, such alterations replace an amino acid with one of similar size and polarity. Preferably the polypeptide is at least 40 %, preferably at least 50 %, more preferably at least 80 % and most preferably at least 90 % homologous with the wildtype growth hormone sequence. In a particularly preferred embodiment the polypeptide is identical with the wild type sequence except for a replacement of the aspartate residue at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 of the sequence listing or at the corresponding position in other growth hormone sequences. Preferred modifications of the amino acid sequence, in addition to the replacement at position 193 of the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2 or at a corresponding position in other growth hormones, are at positions which are not conserved among the vertebrate growth hormones. Conservative substitutions are defined above.

Expression of the recombinant mutant mouse growth hormone was significantly lower than the recombinant wild type mouse growth hormone (Example 9; Fig. 15 + 16). This result appeared to confirm the observation of Cunningham & Wells (1989; Science, 244: 1081-1085) in their alanine scanning mutagenesis study of human growth hormone, which attempted substitution with alanine of aspartate residue 169. In the numbering system of the present invention this aspartate residue is located at position 195 of the human growth hormone (and corresponding to position 193 of the mouse growth hormone in the present invention). The

aspartate to alanine substitution resulted in remarkably poor expression of the alanine mutant protein which prevented Cunningham & Wells from including in their study proteins with a mutation at this particular residue. (note also Cunningham *et al.*, 2000; US 6,022,711). Thus, in addition to this residue not being implicated in binding of GH to the GH receptor, notably in the detailed human studies (see Cunningham & Wells, 1989; Science, 244: 1081-1085. Strasburger, Horm. Res. 1994; 41 (suppl.2): 113-120), but the prior art discouraged investigation of this particular amino acid residue in the human growth hormone as a potential site for investigation of possible antagonists. It can be seen from the expression and purification analysis of the recombinant growth hormones of the present invention (Example 9, Fig. 15 + 16) that the corresponding glycine substitution in human GH is expressed slightly less strongly than the wild type human GH in the bacterial expression system employed. Given that glycine is a conservative replacement for the alanine that performed so poorly in the Cunningham studies, it is most surprising that significant over-expression of both the wild type and mutant recombinant human GH was nevertheless achieved.

The modified growth hormone of the invention is particularly useful for the prevention, treatment and amelioration of medical conditions in which it is important that growth hormone releasing hormone does not counteract the effects of treatment of over-expression or over-activity of growth hormone with a growth hormone analogue. As described in Example 4, although levels of IGF-1 are strongly reduced in the plasma of heterozygous and homozygous mutant animals, the level of GH was comparable to wild type, albeit somewhat lower. Thus the mutant GH was capable of acting appropriately in the GH feedback loop to inhibiting secretion of growth hormone releasing hormone (GHRH), even it demonstrated inactivity and antagonism of other, clinically significant GH effects, as detailed herein.

The present invention also provides a diagnostic method for growth hormone activity deficiency. Patients' peptide material, particularly that in or from blood, serum or plasma, is subjected to analysis for one or more of the amino acid sequences of the present invention. The peptide material may be analysed directly or after extraction, isolation and/or purification by standard methods.

In one embodiment of the invention, the diagnostic method comprises the identification of the modified growth hormone, whereby the modification is associated with the replacement of an amino acid at a position corresponding to position 193 in the amino acid sequence shown in

SEQ ID NO:1 and SEQ ID NO:2 . Such diagnostic methods include those employing detection of the modified growth hormone by its failure to activate a biological pathway (such as that leading to IGF-1 production and secretion; other examples include depletion of proteins identified as being under-expressed in the mutant animal, such as those proteins identified in Fig. 9 and 10). The diagnostic methods of the invention also include those employing detection of the modified growth hormone by its activity in competing with and blocking the action of native growth hormone. Methods of identifying the modified growth hormone include, but are not restricted to any methods known in the art which are able to identify altered conformational properties of the amino acid sequence of the present invention compared to those of the wild type growth hormone. Examples include the specific recognition of the modified protein by other proteins, particularly antibodies; individual or combined patterns of amino acid sequence digestion by known proteases or chemicals. An additional, similar embodiment, the method exploits the failure of another protein to recognize the modified protein, examples being antibodies directed to an epitope of wild type GH that incorporates residue 193 of SEQ ID NO:2, and GH receptors in which this portion of the molecular surface of wild type GH is recognized or in volved in GH binding or activation.

In a further embodiment of the present invention, the principle of the diagnostic method is the detection of a nucleic acid sequence encoding the modified growth hormone of the invention. This includes, but is not restricted to any methods known in the art using nucleic acid hybridizing properties, such as Northern blot, Southern blot, nucleic acid (genomic DNA, cDNA, mRNA, synthetic oligonucleotides) standard methods employing microarrays, and patterns of nucleic acid digestion by known restriction enzymes, preferably *PpuM*I and, in a particularly preferred embodiment, *Ava*II.

The amino acid sequences of the present invention may be made by using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis – techniques for which are well known in the art – or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known and include, for example, M13 mutagenesis. Manipulation of DNA sequences to produce variant proteins which manifests as substitutional, insertional or deletional variants are conveniently described, for example, in Sambrook *et al.* (1989;).

The present invention also provides nucleic acid sequences encoding the above described modified growth hormones. Preferably, the nucleic acid sequences of the invention hybridize with a wild type nucleic acid sequence encoding growth hormone, and/or have at least 40 %, preferably at least 50 %, more preferably at least 80 % and most preferably at least 90 % sequence homology to a wild type nucleic acid sequence encoding growth hormone, in particular the nucleic acid sequence shown in SEQ ID NO:1. In a further preferred embodiment the nucleic acid sequence of the present invention is degenerate with respect to the wild type nucleic acid sequence.

The nucleic acid sequences encoding growth hormone of the invention may exist alone or in combination with other nucleic acids as, for example, vector molecules.

Standard methods are known in the art that may be used in conjunction with the polynucleotides and of the invention and methods described herein to produce a transgenic animal expressing a modified growth hormone of the invention.

Other features and advantages of the invention will be apparent from the following examples.

Example 1: Production of the animals of the invention by ENU-mutagenesis

ENU treatment and breeding strategies

To produce mouse mutants, a C3HeB/FeJ male mouse (The Jackson Laboratory, Bar Harbor ME, U.S.A.) was injected intraperitoneally three times in weekly intervals between 8–10 weeks of age with the highly mutagenic agent ethyl-nitroso-urea (ENU) (Serva Electrophoresis GmbH, Heidelberg, Germany) at 90mg/kg body weight. 50 days after the last injection the injected male mouse was regularly mated to wild type C3HeB/FeJ female partners. The progeny (up to 100 offspring) were then analyzed for phenotypes of dominant traits by body weight and length measurement.

First phenotyping identification

Using a polynomic regression model, a standard growth curve for wild type inbred C3HeB/FeJ mice (The Jackson Laboratory, Bar Harbor ME, U.S.A.) was calculated. The data set included n=10305 male and n=9008 female values of animals aged 10 to 227 days. The resulting

equations for mean body weight of male and female mice at a given day (t) of age is, respectively:

males: $f(t)=9.487025+1.4385603xt-0.021653xt^2+0.00015285xt^3-0.0000003937xt^4 \pm 2x(1.26865119+0.02151744xt)$

females: $f(t)=1.532645+0.688995xt-0.008334xt^2+0.000054345xt^3-0.0000001302xt^4 \pm 2x(1.26865119+0.02151744xt)$.

From three to nine weeks of age mice were weighed every other week. Mice were considered to have an abnormal age-and sex-matched body weight when the weight value deviated more than 2 standard deviations (v<-2) from the mean of the standard animal growth curve. With this strategy, a male of the ENU-injected C3HeB/FeJ male offspring was identified as to have a reduced body weight, and length, and was therefore named SMA1 (for small 1) (Fig 1, 2 and 3: -/+ SMA1 mutant versus +/+ wild type animal).

To confirm the dominant genetic transmission of the reduced body weight and length, the transmission of these traits was analyzed in the progeny of this C3HeB/FeJ SMA1. These 15 to 20 offspring mice were kept in a non-SPF animal facility, on a 12h:12h light-dark cycle (lights on: 6:00 MEZ) at an ambient temperature of 23±2°C (relative humidity: 40-50%) and fed with Altromin 1314 standard chow breeding diet *ad libitum*. Offspring mice were weaned at 21 days and housed in groups of 2-4 individuals of the same sex. Cages were supplied with wood shavings and tissue paper. Animals in individual cages were distinguished with tattoos formed by subcutaneous injection of acrylic paint into the tail base.

The body weights of n= 43 (24 males, 19 females) C3HeB/FeJ SMA1x C3HeB/FeJ wild type offspring and n=14 (6 males, 8 females) wild type C3HeB/FeJ xC3HeB/FeJ offspring were monitored every 7±1 days from weaning to day 119 (week 15) using an electronic balance (Kern 449-47, d=±0.1g) (Fig. 2). Three additional animals (1 male, 2 female) obtained from SMA1 matings had been removed from the experimental group on day 42 for breeding purposes. On day 56±1 (week 8), length measurements of body (=nose to anus distance in millimeters, d=±1mm) and tail (=anus to tail tip distance in millimeters, d=±1mm) were taken from mice anaesthetized with halothane (Fluothane, Zeneca) and placed in a dorsal extended position. Mice were then turned ventrally and head (=nose to occipital distance in millimeters) and tarsus length (right back foot) were measured using a calliper rule (d=±0.1mm). All length

parameters were determined on each of the offspring mice. All measurements were carried out between 8:30 and 11:00 MEZ (Fig. 3 and 4).

From 28 days of age, body weights of male and female SMA1 F1-offspring showed a distinct bimodal distribution pattern with two distinguishable subgroups clustering at a low and a normal body weight compared to wild type offspring (Fig 2). Mice were phenotyped SMA1 if body weight on day 42 was more than two standard deviations lower than the group mean of the standard curve. Using this method, 20 out of 43 (46.5%) SMA1 mutants were detected. (When the 3 animals removed from weight monitoring on day 42 are included in the frequency analysis the number of mutants increases to 22 out of 46 offspring obtained (47.8%).) These values, very close to the 50 % expected from the Mendelian distribution pattern of phenotypes derived from a dominant allele, confirm the dominant inheritance of a SMA1 genetic trait, with almost 100 % penetrance. The weight distribution pattern remained bimodal until day 63, 77 and 105 respectively, when three male mice initially phenotyped mutant had successively increased their body weights to wild-type ($v \geq -2$) range. This increase in body weight was due to an age-related excessive accumulation of fat.

Length measurements revealed that all animals phenotyped SMA1 on day 42 did not only weigh less but were also substantially smaller in size than their wild type littermates and the wild type F1-offspring control group. This finding supports the idea that the low body weight of SMA1 ($v < -2$) is the result of a mutant allele affecting body size rather than body weight itself.

The number of confirmed SMA1 mutants was then expanded by breeding with C3HeB/FeJ wild type partners to establish a colony of this mutant line on an inbred homogeneous C3HeB/FeJ background.

Example 2: Metabolic and physiological characteristics of the mutant animals

Metabolic measurements

Measurements of resting metabolic rate were carried out in an open system as described by Heldmaier & Ruf (1992; J. Comp. Physiol. B, 162: 696-706) using a paramagnetic O_2 analyser (S3AII, Ametek, Paoli PA, U.S.A.) and CO_2 analyser (Advance Optima, Hartmann & Braun, Eschborn, Germany). Both are 2-channel instruments ($d=\pm 0.001$Vol%) allowing direct

comparison of air entering and leaving a plastic chamber (1.8 l) in which a mouse was placed. Analogue output of the recordings was monitored by computer and converted to metabolic.rate (MR) according to the following equation:

$$MR[ml O_2 x h^{-1}] = \Delta Vol\% O_2 x flow[l x h^{-1}] * 10.$$

The set-up allowed parallel measurement of 6 mice, with registration of individual O_2-consumption and CO_2-production for one minute in six minute intervals.

Resting metabolic rate (RMR) was determined in 45 C3HeB/FeJ wild type x C3HeB/FeJ SMA1-offspring at an ambient temperature of 28°±0.5C. Preliminary experiments had shown that this ambient temperature was close to the thermoneutral zone of both SMA1 and wild type mice. At the time of the experiments, mice were 19-21 weeks of age and had been caged singly for at least two weeks. Measurements were always carried out from morning to midday during the resting phase of mice. Animals were allowed to adapt to the metabolic chambers for one hour prior to registration and were not given any food or water during the measurement period. RMR of individual mice was obtained by calculating the mean value of minimal O_2-consumption and the two adjacent data points measured during a three hour registration period (25-34 data points per mouse). Mice were weighed before and after the experiment using an electronic balance (Sartorius 1401MP, Sartorius AG, Goettingen, Germany; d=±0.1g).

On a whole animal basis, RMR at 28°C was lower in phenotypically SMA1 animals than in their wild type littermates (males: 27.99±3.59 ml O_2 (n=14) versus 37.03±4.50 ml O_2 (n=10); females: 25.40±3.54 ml O_2 (n=8) versus 38.71±3.36 ml O_2 (n=13); values provided are mean ± standard deviation). These results suggest that RMR may be reduced proportionally to body size in mutants compared to wild type.

Rectal temperature

At the end of metabolic measurements, before animals were returned to their cages, rectal temperature was determined by inserting a thermal probe (C 856-1, Ahlborn; Ø=2mm) attached to a thermistor (2241-NTC, Ahlborn; d=±0.1°C) 15 mm into the rectum. The temperature readings were taken within 30 seconds after insertion of the probe at a time when stable values had established for at least 10 seconds.

Rectal temperature in mice that had been kept in 28°C for four hours was similar in wild type and SMA1 littermates. Mean rectal temperature measured 35.3±0.7°C in wild type animals (n=23) and 35.1±0.8°C in SMA1 mice with the phenotype (n=22).

Body fat content

Body composition was determined in a subset of 26 mice (12 wild type and 14 SMA1 heterozygous animals). The carcasses were dried to constant weight at 55°C and fat content was determined by extraction of lipids using a Soxhlett apparatus.

As illustrated in Figure 5, body fat content was found to be elevated 4.5-fold (95% confidence interval: 3.0-4.0) in SMA1 compared to wild type mice. Interestingly, the gender-specific differences in fat content, *i.e.* the slightly higher proportion of body fat in wild type females compared to wild type males, is not observed in SMA1 mice.

Example 3: Necroscopy and organ histology of the mutant animals

Compared to wild type mice, the SMA1 mice have an increased ratio between the body weight and the length of tibia. They also exhibit a thinner cortical bone and smaller skeletal muscle fibers. Besides a reduced size no other abnormalities were observed in endocrine organs such as the thyroid gland, pancreas, and adrenal glands. Testicles showed active spermatogenesis and signs of sexual dimorphism were present in the submandibular gland. In contrast, the glomeruli had a female type of Bowman's capsule that suggests some deficiency of sexual hormones. Eosinophilic inclusions were found in the arcuate nucleus of the hypothalamus.

In the pituitary gland the adenohypophysis looks smaller than normal in relation to the neurohypophysis (Fig. 8). Microscopic analysis performed on cross-sections through the anterior, intermediate and posterior lobes of the pituitary gland with a classical hematoxylin/eosin and PAS staining of the section, revealed hypoplasia of the adenohypophysis. The pituitary gland has a normal architecture with well-defined neurohypophysis and adenohypophysis, but the photographs clearly show that the anterior lobe of the adenohypophysis is smaller than the control adenohypophysis whereas the neurohypophysis has a normal size. Under higher magnification acidophilic cells of the adenohypophysis, normally numerous and readily identified in control mice due to their

strongly acidophilic granules in cytoplasm (cells marked with an asterisk) are not detected in the adenohypophysis of the mutant animal indicating either that acidophilic cells which secrete growth hormone or prolactin are missing or do not have secretion granules.

Example 4: IGF-1 and GH plasma levels in mutant animals of the present invention and partial differential analysis of the liver proteome

Blood sampling

EDTA-blood samples of three-month old mice which had fasted overnight were taken by puncture in the retro-orbital sinus under ether anesthesia, using heparin-coated capillaries and EDTA-coated collection tubes.

IGF-1 plasma concentration analysis was performed on 10µl of undiluted EDTA plasma collected as described above, using an IGF-1 Active rat IGF-1 EIA from DSL as described in the manufacturer's instructions. The optical density values were read from a 96-well reaction plate using a MTP Reader MRX II (Dynex Technologies / Thermo Labsystems, Helsinki, Finland). The results shown in Fig. 6 clearly demonstrate that C3HeB/FeJ-SMA1 heterozygous mutant mice, have lower plasma levels of IGF-1 (~ 50-100 ng/ml) as compared to the C3HeB/FeJ wild type mice or C3HeB/FeJ-SMA1 x C3HeB/FeJ-wild type descendant mice without the phenotype (~ 150-200 ng/ml).

The measurement of GH plasma levels was performed on 25µl of 1:5 in diluted (in the EIA kit sample buffer) plasma samples using a growth hormone EIA from Amersham Pharmacia Biotech (Freiburg, Germany) as indicated in the manufacturer's instructions. The optical density values were red out on a 96-well reaction plate using a MTP Reader MRX II (Dynex Technologies). The results shown in Fig. 7 clearly demonstrate that C3HeB/FeJ-SMA1 heterozygous mutant mice have a subnormal to normal GH plasma levels as compared to C3HeB/FeJ wild type mice or C3HeB/FeJ-SMA1 x C3HeB/FeJ-wild type descendant mice without the phenotype.

2D-Gel Electrophoresis of Liver Samples

Samples were prepared from liver tissue taken from an 85 day old homozygous SMA1 female, and from a 93 day old wild type C3H female mouse. Frozen liver (250 mg) as homogenized in a liquid nitrogen cooled mortar and the powder suspended in 1 ml lysis buffer (9M urea, 2%

CHAPS, 1% DTT, 0,8% Pharmalyte, 4 mM Pefabloc). The protein concentration of the lysate was determined with the Bio-Rad Protein Assay according to the manufacturer's instructions.

Two dimensional electrophoresis: 500 µg, 600 µg or 800 µg of liver proteins were loaded on IPG strips 6-9, 5.5-6.7 or 4.5-5.5, respectively. Isoelectric focusing was carried out in the IPG Phor apparatus of Amersham Pharmacia with total Volthours of 53000 for IPG 6-9 and 60000 for IPG 4.5-5.5 and IPG 5.5-6.7. The second dimension separation was performed on 12% SDS polyacrylamide gels in an ISO-DALT apparatus (Hoefer). Coomassie staining was performed with the Colloidal Blue Stain from Novex according to the manufacturer's instructions. Results are illustrated in Fig. 9 and 10.

Example 5: mapping and cloning of the mutation in the mutant animals of the present invention

For chromosomal mapping of the mutant loci in the SMA1 mutant line a standard outcross/backcross strategy in strain C57Bl/6Jico (Ifacredo, France) was used in combination with microsatellites markers specific for the C3HeB/FeJ and C57Bl/6Jico strains.

DNA Isolation from rodent tails

The genomic DNA was purified from 1 cm long piece of tails of wildtype C3H, and C57BL/6J mice, as well as from C3HeB/FeJ-*Sma1*$^{+/-}$, C3HeB/FeJ-*Sma1*$^{-/-}$, and [(C3HeB/FeJ- *Sma1*$^{+/-}$ x C57Bl/6Jico) x C57Bl/6Jico] F_2R mutant mice by using the "DNeasy 96 Tissue Kit" (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Genotyping of mice by polymerase chain reaction (PCR)

To genotype the F_2R progeny microsatellite markers specific for C3HeB/FeJ and C57Bl/6Jico were used. Oligonucleotides were obtained from MWG BIOTECH (Ebersberg, Germany). PCR reactions were performed with fluorescent-labeled primers, whose sequences were taken from Schalkwyk *et al.*, 1999, http://www-genome.wi.mit.edu/ (site maintained by the Whitehead Institute for Genome Research, Cambridge MA, U.S.A.). The PCR reactions were performed, in a MJ tetrad thermocycler PTC 225 device (MJ Research, Inc., Waltham MA, U.S.A.) in 10 µl reaction volumes using Pharmacia *Taq*-DNA polymerase (Amersham Pharmacia Biotech, Piscataway NJ, USA). A four minute denaturation step was then followed

by 28 amplification cycles comprising each 30 sec denaturation and 30 sec annealing at the respective temperatures given in Schalkwyk *et al.*, 1999, and 30 sec extension at 72°C. Samples were amplified with different dyes and products were separated on an ABI 377 DNA sequencing device (PE Applied Biosystems, Foster City, USA) using internal length standards in every lane. Analysis was performed with Genescan version 3.0 and Genotyper version 2.1 software from ABI. The analysis of 48 [(C3HeB/FeJ- $SMA1^{+/-}$ x C57Bl/6Jico) x C57Bl/6Jico] mice allowed the assessment that the SMA1 mutation coseggregates with the marker D11Mit23 on chromosome 11 (Fig. 11A). Furthermore, a detailed haplotype analysis of these animals using further microsatellite markers located in this region of chromosome 11 to refine the mapping, demonstrated that the mutation was located between microsatellite markers D11Mit333 and D11Mit301, a locus where the growth hormone gene is located (Fig. 11B).

PCR Amplification of Growth Hormone

PCR conditions were as previously described. The nucleotide sequence of the *Mus musculus* growth hormone gene and its promoter were taken from GenBank/EMBL (Accession Number Z46663). The growth hormone specific primers listed below were designed by "DOPE interactiva" (http://doprimer.interactiva.de/; Interactiva, Ulm, Germany) and set up in 25 ul PCR reactions carried out on 10 ng of genomic DNAs using the Pharmacia Taq-DNA polymerase (Amersham Pharmacia Biotech,NJ, USA) for 28 amplification cycles consisting of 30 sec denaturation at 94°C, 30 sec annealing at 55°C, and 90 sec extension at 72°C.

11M-Gh-F2: TCGGACCGTGTCTATGAGAAA; (SEQ ID NO:5)
11M-Gh-R2: GCTTCCAGGAACAAGATTGACA; (SEQ ID NO:6)
11M-Gh-F3: TAAGAGATCTAGCCACAGGGA; (SEQ ID NO:7)
11M-Gh-R3: CACTGCTGTTGGGAAAAGAAAG; (SEQ ID NO:8)
11M-Gh-F5: TCCTACCCTTGGATTCAAAA; (SEQ ID NO:9)
11M-Gh-R5: ACCAGCTTGTGTCTCGTCA; (SEQ ID NO:10)
11M-Gh-F6: CCGTTTGTGGAAGCAGGAA; (SEQ ID NO:11)
11M-Gh-R6: ATAACCCCAGGCTAGTCCAT; (SEQ ID NO:12)
11M-Gh-F8: GACAGTGCCCTCTAGTGCTCAGTG; (SEQ ID NO:13)
11M-Gh-R8: TTATCGTCTCATCGCCACCTTTGC; (SEQ ID NO:14)

DNA Sequencing

PCR amplicons were purified by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturers protocol. PCR products were sequenced using

forward/reverse PCR primers and "Big Dye" thermal cycle sequencing Kit (ABI PRISM, Applied Biosystems, Foster City CA, U.S.A.). The reaction products were analyzed on an ABI 377 DNA sequencing device.

Sequence Analysis

The sequences were edited manually and contig assembly for mutation detection was performed using Sequencher version 4.0.5 (Gene Codes Corp., Ann Arbor MI, U.SA.). The sequence analysis, as shown by the chromatogram in Fig. 11C, revealed (at the location corresponding to position 639 of the nucleic acid sequence shown in SEQ ID NO:1) the presence of an A to G transition in the 5th exon of the Gh gene in C3HeB/FeJ-SMA1 homozygous and heterozygous mutant animals and in C3HeB/FeJ wild type mice. This transition leads to an Asp to Gly amino acid transition at position 193 (positions are as described in the amino acid sequence shown in SEQ ID NO:1 and SEQ ID NO:2. This Asp residue, located in the C-terminal helix domain is very conserved between species (Fig. 13).

Example 6: Method for production of the mutant animals of the present invention by gene targeting technology

The λKOS-System, a yeast-bacteria shuttle system (Wattler *et al.* 1999; Biotechniques, 26: 1150-1159) is used to construct a recombinant vector to insert a point mutation at nucleotide 128 of the fifth exon of the mouse growth hormone gene Gh. A clone containing the full length Gh coding sequence flanked by further 1,5 kb of downstream sequences (Fig. 14) is isolated from the λKOS library by hybridization with a probe corresponding to the PCR product obtained with the following primer set: C-5'-gaggatggactagcctggg-3' (SEQ ID NO:22) and D-3'-cctgtcgtgggaaagaaggg-3' (SEQ ID NO:23), both primers amplifying a 150 bp long PCR product from the fourth intron. The clone was converted into a plasmid by transduction of a bacterial strain which expresses the Cre recombinase (Stratagene BNN132, Stratagene, La Jolla CA, U.S.A.). The following oligonucleotides were then used to insert the point mutation at nucleotide 128 from the fifth exon of the mouse growth hormone gene Gh.

Oligonucleotide 1 matches to the nucleotides at positions 159 to 106 of the fifth exon of mouse growth hormone gene Gh, with C at position 128 instead of the G of the wild type sequence: 5'-gacccgcaggtaggtctccgctttgtgcaggcccttcttgaagcaggagagcag-3' (SEQ ID NO:15).

Oligonucleotide 2 matches to the nucleotides at positions 103 to 125 from the fourth intron of mouse growth hormone gene Gh, with a Sfi restriction site at the 5' end (shown in bold): 5'-gc**gggccgtagcggcc**gggaggcacagctcccgagtctcc-3' (SEQ ID NO:16).

Oligonucleotide 3 matches to the nucleotides at positions 103 to 78 from the fourth intron of mouse growth hormone gene Gh, with a Sfi restriction site at its 5' end (shown in bold type-face): 5'-gc**gggccacgcaggcc**tccaccccaggaccgaaggaaaagcc-3' (SEQ ID NO:17).

Oligonucleotide 4 matches to the nucleotides at positions 3 to 29 from the fourth intron of mouse growth hormone gene Gh: 5'-gaggatggactagcctggggttatgcc-3' (SEQ ID NO:18).

Oligonucleotides 1, 2, 3 and 4, were set in PCR reactions to amplify two fragments, named A and B, respectively, which were cleaved by Sfi after purification by QIAquick kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and then ligated to a yeast/bacteria-selection cassette (Wattler *et al.* 1999; Biotechnques, 26: 1150-1159). Wild type yeast were then transformed with the ligation product (A + B + yeast/bacteria selection cassette) and the pKOS-clone together and a double selection is performed by plating onto defined growth medium lacking uracil and tryptophan. The only yeast able to survive are those carrying a homologous recombination between the selection cassette with the ligated fragment A + B + yeast/bacteria selection cassette and the genomic pKOS-clone DNA. Single yeast colonies were propagated and tested for the presence of the point mutation by colony-PCR with the oligonucleotides 5 and 6 as 5' and 3' primers, respectively.

Oligonucleotide 5 corresponds to the nucleotides at positions 203 to 180 from the fifth exon of mouse growth hormone gene Gh: 5'-ggatgaaggcacagctgctttcc-3' (SEQ ID NO:19).

Oligonucleotide 6 corresponds to the sequence CAT-1: 5'-tcatcatgccgtctgtgatg-3' from the bacterial resistance gene (SEQ ID NO:20).

PCR products were subsequently sequenced using oligonucleotide 5 as a primer. The DNA of the colonies whose product contains the point mutation was isolated and used to transform *E. coli* further grown on (amp/cat) selection medium. The transformation of mouse 129 ES cells with the final vector construct was performed according to standard procedures. Resulting ES-cell clones were double selected with G418, positive and FIAU, negative selection. To identify

positive targeted clones, ES-cell DNA was digested and hybridized with a 5'-external probe as depicted in Fig.14. A single integration was verified by hybridization of the same Southern blot with a probe generated from the selection cassette, as outlined in Fig. 14.

Moreover, the presence of the point mutation was examined by PCR using oligonucleotide 5 and a primer with the nucleotide sequence (5'-gcagcgcatcgccttctatc-3': SEQ ID NO:21) from the neo-selection cassette, followed by PCR product sequencing using oligonucleotide 5. Clones containing the point mutation were used to produce chimeric mice by blastocyst injection and transfer using standard methodology, well known in the art.

The chimeras were bred to wildtype mice and in the following generation the same southern strategy as outlined above was used to determine germline transmission. By breeding of the heterozygous mice with a transgenic lines bearing the Cre-recombinase under the control of the protamine promoter, compound heterozygotes were generated, which were heterozygous for cre as well as for the mutation. Resulting males were bred with wildtype females and the litters were analyzed for the presence of cre-recombination by a PCR approach as follows. The primer combination 4 and 5 will result in an informative bandshift. A wildtype animal will result in a 390 bp PCR product, while a recombined heterozygotic animal will show a 423 bp product which includes the one loxP-site which is left in the genome after Cre recombination, and, additionally, the wildtype band. Without cre-recombination the selection cassette (2.1 kb) is amplified.

Example 7: Method for the detection of the nucleic acid of the present invention

Mutation detection using a polymorphic *Ava* II restriction site.

The nucleotide transition A to G in the nucleic acid sequence of the growth hormone results in the disappearance of a restriction site for the two restriction enzymes *Ava*II and *PpuM*I. As a result, the restriction pattern of the nucleic acid sequence of growth hormone will differ between the wild type and the mutant sequence which is not recognized anymore by the restriction enzymes. This procedure was applied on amplified fragments from nucleic acids extracted from mutant and wild type mice as already described in the example. The PCR and restriction steps were performed as follows:

The PCR reactions were carried out on 10ng genomic DNAs using primers 11M-Gh-F2 (SEQ ID NO:5) and 11M-Gh-R2 (SEQ ID NO:6). PCR reactions were performed in 25 µl using *Taq*-DNA polymerase (Amersham Pharmacia Biotech, NJ, USA) according to the manufacturer's protocol. A further 4 minute denaturation step was followed by 28 cycles of denaturation at 94°C for 30 sec; annealing at 55°C for 30 sec; and 90 sec extension at 72°C. For the enzymatic digestion, 7 µl of PCR reaction, 10 µl H_2O, 2 µl NEB buffer 4 and 1 unit *Ava*II (New England BioLabs Inc., Beverly MA, USA) were incubated at 37°C for 2 hours. Restriction fragments were size separated by electrophoresis on 2% agarose gels (SeaKem ME agarose, Biozym Diagnostik Gmbh, Hess. Oldenburg, Germany) with 0.5 µg ethidium bromide ml^{-1} agarose. Gels were photographed after 2 hours electrophoresis at 3 V m^{-1}. As shown in Fig. 12, the presence of the point mutation deletes one of the *Ava*II sites and one of the *PpuM*I sites, present in the PCR product of the wild type. This results in the appearance of a longer restriction fragment in the SMA1 mutant samples, that is a restriction polymorphism (Fig. 12). This procedure even allows the differentiation between heterozygous and homozygous SMA1 mutants, as the remaining small fragment produced by the additional *Ava*II digestion of the heterozygous samples does not appear in samples from homozygous mutants.

Example 8: Cloning of mouse and human growth hormones into expression vectors

Cloning of human GHwt into mammalian expression vector pEAK8

First strand cDNA synthesis was performed using human pituitary gland polyA+ RNA (product 6584-1, Clontech Laboratories, Inc., Palo Alto CA, USA), primed with oligo hGH-2 (SEQ ID NO:24). Amplification was by polymerase chain reaction (PCR) with human GH specific primer hGH-1 (SEQ ID NO:25) and hGH-4 (SEQ ID NO:26), amplifying a 769 bp product of human GH, including 55 bp 5' UTR sequence, 78 bp signal peptide encoding sequence, 576 bp mature peptide encoding sequence and 60 bp 3' UTR sequence (SEQ ID NO:29). SEQ ID NO:30 is the encoded amino acid sequence corresponding to nucleic acid sequence SEQ ID NO:29. PCR was performed with blunt end-generating *Pwo* proof-reading polymerase. This 769 bp PCR product was sub-cloned into *Eco*RV-linearized (blunt) pCR2.1-TOPO vector (product K4500-01, Invitrogen Life Technologies, Inchinnan, Scotland), creating pCR2.1-TOPO hGHwt, which was then subjected to restriction digestion by *Eco*R1 and *Not*I. The *Eco*R1 / *Not*I insert was isolated and sub-cloned into mammalian expression

vector pEAK8β-gal that had been predigested with *Eco*R1 / *Not*I, to generate construct pEAK8β-gal-hGHwt.

Cloning of human GHmut into mammalian expression vector pEAK8

Primers hGH-9 (SEQ ID NO:27) and hGH-10 (SEQ ID NO:28) were used with the QuickChange site-directed mutagenesis kit (product 200518, Strategene, La Jolla CA, USA) according to the manufacturer's instructions to introduce the point mutation (A to G transition) at position +584 in the hGH cDNA. The DNA template was vector pCR2.1-TOPO hGHwt. The final clone after mutagenesis was named pCR2.1-TOPO hGHmut (SEQ ID NO:31). This clone, pCR2.1-TOPO hGHmut, was subjected to restriction digestion with *Eco*RI and *Not*I, the *Eco*RI / *Not*I hGHmut insert was isolated and sub-cloned into mammalian expression vector pEAK8β-gal that had been predigested with *Eco*R1 / *Not*I, to generate construct pEAK8β-gal-hGHmut. The amino acid sequence SEQ ID NO:32, which is encoded by the DNA sequence of SEQ ID NO:31, exhibits a D to G transition at position 195.

Cloning of mouse GHwt and mouse GHmut into mammalian expression vector pEAK8

First strand cDNA synthesis was performed using either (a) C3H wild type mouse pituitary gland total RNA, or (b) SMA1 mouse pituitary gland total RNA, primed with oligo mGH-3 (SEQ ID NO:33). Amplification was by polymerase chain reaction (PCR) with mouse GH specific primer mGH-1 (SEQ ID NO:34) and mGH-10 (SEQ ID NO:35), amplifying a 718 bp product of mouse GH, including 52 bp 5' UTR sequence, 78 bp signal peptide encoding sequence, 573 bp mature peptide encoding sequence and 15 bp 3' UTR sequence. PCR was performed with blunt end-generating *Pwo* proof-reading polymerase. The 718 bp PCR product was sub-cloned into *Eco*RV-linearized (blunt) pCR2.1-TOPO vector, creating respectively (a) pCR2.1-TOPO mGHwt (SEQ ID NO:36; SEQ ID NO:37 is the encoded amino acid sequence corresponding to nucleic acid SEQ ID NO:36), or (b) pCR2.1-TOPO mGHmut (SEQ ID NO:38) carrying the point mutation (A to G transition) at position at the mGH cDNA. These amplification products were then subjected to restriction digestion by *Eco*R1 and *Not*I. The *Eco*R1 / *Not*I insert was isolated and sub-cloned into mammalian expression vector pEAK8β-gal that had been predigested with *Eco*R1 / *Not*I, to generate constructs (a) pEAK8β-gal-hGHwt, or (b) pEAK8β-gal-hGHmut, respectively. The amino acid sequence SEQ ID NO:39, which is encoded by the DNA sequence of SEQ ID NO:38, exhibits a D to G transition at position 193.

Cloning of mouse GHwt and mouse GHmut into bacterial expression vector pQE30

Expression vector constructs (a) pEAK8β-gal-mGHwt and (b) pEAK8β-gal-mGHmut were amplified separately by PCR using the primers mGHfw-*Hin*d (SEQ ID NO:40) and mGHrev-*Hin*d (SEQ ID NO:41). Each procedure generated a DNA product of 573 bp: (a) SEQ ID NO:42 (mGHwt) and (b) SEQ ID NO:44 (mGHmut), respectively. (SEQ ID NO:43 is the is the encoded amino acid sequence corresponding to nucleic acid sequence SEQ ID NO:42.) Each of these products was subjected to restriction digestion by *Hin*dIII and then sub-cloned into *Hin*dIII-linearized pQE30 vector (product 32915, Qiagen, Hilden, Germany) to generate the constructs (a) pQE30-mGHwt and (b) pQE30-mGHmut, respectively. The amino acid sequence SEQ ID NO:45, which is encoded by the DNA sequence of SEQ ID NO:44, exhibits a D to G transition at position 164.

Cloning of human GHwt and human GHmut into bacterial expression vector pQE30

Expression vector constructs (a) pEAK8β-gal-hGHwt and (b) pEAK8β-gal-hGHmut were amplified separately by PCR using the primers hGHfw-*Hin*d (SEQ ID NO:46) and hGHrev-*Hin*d (SEQ ID NO:47). Each procedure generated a DNA product of 576 bp: (a) SEQ ID NO:50 (hGHwt) and (b) SEQ ID NO:48 (hGHmut), respectively. Each of these products was subjected to restriction digestion by *Hin*dIII and then sub-cloned into *Hin*dIII-linearized pQE30 vector to generate the constructs (a) pQE30-hGHwt and (b) pQE30-hGHmut, respectively. The amino acid sequence SEQ ID NO:49, which is encoded by the DNA sequence of SEQ ID NO:48, exhibits a D to G transition at position 167.

Example 9: Purification of recombinant mouse and human growth hormones

Expression of recombinant mouse and human growth hormones

E. coli M15 cells containing recombinant expression plasmids for recombinant mouse and human growth hormone were grown individually in Luria-Bertani (LB) medium in the presence of kanamycin (25µg/mL) (Sigma, St. Louis, USA) and ampicillin (100µg/mL) (Roth, Karlsruhe, Germany) at 25°C. The recombinant peptides expressed by these plasmids were: recombinant mouse growth hormone, wild type (r-mGHwt); recombinant mouse growth hormone SMA1-Gly_{193} mutant sequence (r-mGHmut); human growth hormone, wild type (r-hGHwt) and recombinant human growth hormone, Gly_{195} mutant sequence (r-hGHmut). The cultures were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) (USB, Cleveland, USA) when they reached OD_{600} = 0.6 (optical density), and were then cultivated

for another 16h. Following lysis of the bacterial cells, synthesis of growth hormone was determined in both supernatant and total extract by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out using the standard method described by Laemmli. A predominant band corresponding to the expected size of GH (27kDa) was observed in total extract and cytosolic fraction of induced cells.

Isolation and purification of r-mGHwt/mut and r-hGHwt/mut.

The cytosolic fraction was prepared by first harvesting the cells at 5000 rpm for 15 min. The pellet was resuspended in chilled Lysis-Buffer (100 mM NaH_2PO_4/Na_2PO_4 pH=8.0; 300 mM KCl, 5 mM imidazole), lysozyme (Sigma Chemical Co., St Louis MO, USA) was added to 1mg/mL and the cell suspension kept on ice for 30 minutes. The cells were sonicated on ice six times for 10 sec with 5 sec pauses between. The cells were re-harvested and the supernatant (cytosolic fraction) was stored for separate analysis and purification. The insoluble aggregates in the inclusion bodies were solubilized in Lysis-Buffer with 8M urea (Merck KGaA, Darmstadt, Germany) added and the aggregate material purified.

Lysate or solubilized aggregate samples containing recombinant growth hormone were loaded individually onto Ni^{2+} agarose (Qiagen, Hilden, Germany) and gently mixed by shaking at 4°C for 60 min. The lysate-Ni-NTA mixture was loaded into a column (Micro Bio-Spin, Chromatography Columns, BIO-Rad Hercules, USA) with bottom outlet capped, allowing resin to settle. The cap was then removed and flow-through material collected. The extract was washed once with Wash-Buffer (100 mM NaH_2PO_4/Na_2PO_4, pH=8.0; 300 mM KCl, 20 mM imidazole), the flow-through collected. The recombinant protein was eluted two times with 0.5 mL of Elution-Buffer (100 mM NaH_2PO_4/Na_2PO_4, pH=8.0; 300 mM KCl, 250 mM imidazole). Fractions were analysed by SDS-PAGE and by immunoblotting with anti-hGH (anti-(human growth hormone)) and anti-mGH (anti-(mouse growth hormone)) rabbit polyclonal sera, the results being presented in Figures 15 and 16.

The SDS-PAGE gel shown in Fig. 15 demonstrates that each of the four types of recombinant growth hormone investigated is expressed in the bacterial inclusion bodies: recombinant mouse growth hormone, wild type (mGHwt); recombinant mouse growth hormone, SMA1 Gly_{193} mutant form (mGHmut); recombinant human growth hormone, wild type (hGHwt); and recombinant human growth hormone, Gly_{195} mutant form (hGHmut). Although the recombinant mGHmut is expressed less vigorously than the other three samples, Fig. 15

clearly demonstrates that all four recombinant growth hormones may be expressed and purified by this method.

The SDS-PAGE gel shown in Fig. 16 demonstrates that both wild type (wt) and Gly_{195} mutant forms (mut) of recombinant human growth hormone (hGH) were over-expressed in the bacterial cells and able to be purified from the lysate supernatant under non-denaturing conditions by this method. The wild type form of recombinant mouse growth hormone (mGH wt) was also over-expressed and purified from the lysate supernatant. The mutant form of recombinant mouse growth hormone (mGH mut) was not over-expressed, being barely detectable in the lysate supernatant.

Claims

1. A non-human animal model expressing a modified growth hormone, whereby the modification is an amino acid substitution in the wildtype growth hormone sequence at the position corresponding to position 193 of the amino acid sequence shown in SEQ ID NO:1.

2. The non-human animal model according to claim 1, whereby the modified growth hormone is derived from a vertebrate, in particular from a mammal.

3. The non-human animal model according to claim 2, whereby the modified growth hormone is derived from bovine, rat or mouse.

4. The non-human animal model according to any one of claims 1 to 3, wherein said amino acid substitution replaces an aspartate residue.

5. The non-human animal model according to claims 1 to 4, wherein said amino acid substitution is by an amino acid selected from the group consisting of alanine, serine, threonine, proline and glycine.

6. The non-human animal model according to claim 5, wherein the modified growth hormone has the amino acid sequence shown in SEQ ID NO:4.

7. The non-human animal model according to any one of claims 1 to 6, whereby the growth hormone sequence further comprises modifications selected from the group consisting of amino acid substitutions, deletions and insertions.

8. The non-human animal model according to any of claims 1 to 7, whereby the animal is from a genus selected from the group consisting of Mus (e.g., mice), Rattus (e.g., rats), Oryctologus (e.g., rabbits) and Mesocricetus (e.g., hamsters).

9. The non-human animal model according to claim 8, whereby the animal is a mouse.

10. The non-human animal model according to any one of claims 1 to 9, whereby the modified growth hormone is encoded by a nucleic acid sequence which is homozygous in said animal model.

11. The non-human animal model according to any one of claims 1 to 10, whereby the animal exhibits one or more of the following phenotypical features:

(i) small proportionate stature characterized by reduced body weight and length, and reduced size of all body parts and organs;

(ii) increased body fat content;

(iii) non-proportionate reduced size and defective histology of the anterior pituitary gland;

(iv) significant concentrations of circulating endogenous GH comprising the sequence of SEQ ID NO:3;

(v) levels of total GH in the plasma that are comparable, but significantly lower than in the wild type animals;

(vi) abnormally low IGF-1 plasma levels;

(vii) reduced O_2-consumption;

(viii) a body temperature that is normal for the wild type animal;

(ix) reduced or defective cognitive functions and information processing capacity.

12. Primary cells and cell lines derived from the animal model according to any one of claims 1 to 11.

13. A modified growth hormone amino acid sequence, whereby the modification is an amino acid substitution in the wild type growth hormone sequence at a position selected from the following group:

(a) a position corresponding to position 193 of the amino acid sequence as shown in SEQ ID NO:1 and SEQ ID NO:2;

(b) a position corresponding to position 195 of the amino acid sequence as shown in SEQ ID NO:29 and SEQ ID NO:30.

14. The growth hormone amino acid sequence according to claim 13, whereby the growth hormone is derived from a vertebrate, in particular from a mammal.

15. The growth hormone amino acid sequence according to claim 14, whereby the growth hormone is derived from bovine, rat or mouse

16. The growth hormone amino acid sequence according to any one of claims 13 to 15, whereby the substitution is by an amino acid residue selected from the group consisting of alanine, serine, threonine, proline and glycine

17. The growth hormone amino acid sequence according to claim 16, whereby the amino acid sequence is as shown in SEQ ID NO:4.

18. The growth hormone amino acid sequence according to claims 13 to 17 further comprising modifications selected from the group consisting of amino acid substitutions, deletions and insertions.

19. A modified growth hormone polypeptide, wherein the modification comprises the modified growth hormone sequence according to claims 13 to 18.

20. The modified growth hormone polypeptide of claim 19, wherein the modified growth hormone sequence is selected from the group of:

(i) a mouse growth hormone sequence;

(ii) a human growth hormone sequence.

21. A modified human growth hormone polypeptide according to claim 20, wherein the modification comprises an amino acid substitution in the wild type growth hormone sequence at the position corresponding to position 195 of SEQ ID NO:32.

22. The modified human growth hormone polypeptide of claim 21, wherein said amino acid substitution is an amino acid selected from the group consisting of serine, threonine, proline and glycine.

23. A protein comprising the polypeptide according to any of claims 19 to 22, wherein the protein is a chimeric protein.

24. A monoclonal antibody capable of binding specifically to the modified growth hormone polypeptide of any of claims 19 or 22 in preference to wild type growth hormone.

25. A composition comprising a polypeptide or protein selected from the group of:

(i) the modified growth hormone polypeptide of any of claims 19 to 22;

(ii) the chimeric protein of claim 23;

(iii) the monoclonal antibody of claim 24;

wherein the composition further comprises a pharmaceutically acceptable carrier.

26. The modified growth hormone polypeptide of any of claims 19 to 22 for the prevention, treatment or amelioration of a medical condition in a mammalian subject, particularly a human subject.

27. A protein selected from the group of:

(i) the chimeric protein of claim 23;

(ii) the monoclonal antibody of claim 24;

for the prevention, treatment or amelioration of a medical condition in a mammalian subject, particularly a human subject.

28. The composition of claim 25 for preventing, treating or ameliorating a medical condition in a mammalian subject, particularly a human subject.

29. Use of the modified growth hormone polypeptide of any of claims 19 to 22 for the manufacture of a medicament for the prevention, treatment or amelioration in a mammal of at least one medical condition selected from the following group:

(i) excessive or undesirable growth rate;

(ii) over-activity or undesirable activity of endogenous growth hormone;

(iii) over-expression, over-production or undesirable production of endogenous growth hormone;

(iv) an excessive or undesirable condition shown to be modulated by endogenous growth hormone;

(v) over-activity or undesirable activity of endogenous IGF-1;

(vi) over-expression, over-production or undesirable production of endogenous IGF-1;

(vii) an excessive or undesirable condition shown to be modulated by endogenous IGF-1 activity;

(viii) excessive or undesirable proliferation of microvascular cells, wherein the proliferation is stimulated by endogenous growth hormone;

(viii) tumorogenesis, wherein the tumour's growth is stimulated by endogenous growth hormone;

(ix) excessive or undesirable serum concentration of cholesterol or low density lipoprotein (LDL);

(x) peripheral neuropathy;

(xi) glomerulosclerosis;

(xii) diabetes;

(xiii) acromegaly, gigantism and associated disorders;

(xiv) excessively lean body mass or a deficiency in body fat content.

30. Use of the protein according to claim 27 for the manufacture of a medicament for the prevention, treatment or amelioration in a mammal of at least one medical condition selected from the following group:

(i) excessive or undesirable growth rate;

(ii) over-activity or undesirable activity of endogenous growth hormone;

(iii) over-expression, over-production or undesirable production of endogenous growth hormone;

(iv) an excessive or undesirable condition shown to be modulated by endogenous growth hormone;

(v) over-activity or undesirable activity of endogenous IGF-1;

(vi) over-expression, over-production or undesirable production of endogenous IGF-1;

(vii) an excessive or undesirable condition shown to be modulated by endogenous IGF-1 activity;

(viii) excessive or undesirable proliferation of microvascular cells, wherein the proliferation is stimulated by endogenous growth hormone;

(viii) tumorogenesis, wherein the tumour's growth is stimulated by endogenous growth hormone;

(ix) excessive or undesirable serum concentration of cholesterol or low density lipoprotein (LDL);

(x) peripheral neuropathy;

(xi) glomerulosclerosis;

(xii) diabetes;

(xiii) acromegaly, gigantism and associated disorders;

(xiv) excessively lean body mass or a deficiency in body fat content.

31. Use of the composition according to claim 25 for the manufacture of a medicament for the prevention, treatment or amelioration in a mammal of a medical condition resulting from the administration of a pharmaceutical composition, particularly wherein said medical condition corresponds to at least one of the medical conditions selected from the following group:

(i) excessive or undesirable growth rate;

(ii) over-activity or undesirable activity of endogenous growth hormone;

(iii) over-expression, over-production or undesirable production of endogenous growth hormone;

(iv) an excessive or undesirable condition shown to be modulated by endogenous growth hormone;

(v) over-activity or undesirable activity of endogenous IGF-1;

(vi) over-expression, over-production or undesirable production of endogenous IGF-1;

(vii) an excessive or undesirable condition shown to be modulated by endogenous IGF-1 activity;

(viii) excessive or undesirable proliferation of microvascular cells, wherein the proliferation is stimulated by endogenous growth hormone;

(viii) tumorogenesis, wherein the tumour's growth is stimulated by endogenous growth hormone;

(ix) excessive or undesirable serum concentration of cholesterol or low density lipoprotein (LDL);

(x) peripheral neuropathy;

(xi) glomerulosclerosis;

(xii) diabetes;

(xiii) acromegaly, gigantism and associated disorders;

(xiv) excessively lean body mass or a deficiency in body fat content.

32. A nucleic acid sequence encoding the growth hormone amino acid sequence according to any one of claims 13 to 18.

33. An isolated polynucleotide capable of encoding the polypeptide of any of claims 19 to 22, or the chimeric protein of claim 23.

34. A vector comprising the polynucleotide of claim 33.

35. A host cell comprising the vector of claim 34.

36. A process for preparing the modified growth hormone polypeptide of any of claims 19 or 22, wherein the process comprises :

(i) growing a culture of the host cell of claim 35 in a suitable culture medium; and

(ii) recovering the modified growth hormone polypeptide from the culture.

37. The isolated polynucleotide of claim 33, or the vector of claim 34, for the prevention, treatment or amelioration of a medical condition in a mammalian subject, particularly a human subject.

38. Use of the polynucleotide of any of claims 33 or 37, or the vector of any of claims 34 or 37, for the manufacture of a medicament for the prevention, treatment or amelioration in a mammal of at least one medical condition selected from the following group:

(i) excessive or undesirable growth rate;

(ii) over-activity or undesirable activity of endogenous growth hormone;

(iii) over-expression, over-production or undesirable production of endogenous growth hormone;

(iv) an excessive or undesirable condition shown to be modulated by endogenous growth hormone;

(v) over-activity or undesirable activity of endogenous IGF-1;

(vi) over-expression, over-production or undesirable production of endogenous IGF-1;

(vii) an excessive or undesirable condition shown to be modulated by endogenous IGF-1 activity;

(viii) excessive or undesirable proliferation of microvascular cells, wherein the proliferation is stimulated by endogenous growth hormone;

(viii) tumorogenesis, wherein the tumour's growth is stimulated by endogenous growth hormone;

(ix) excessive or undesirable serum concentration of cholesterol or low density lipoprotein (LDL);
(x) peripheral neuropathy;
(xi) glomerulosclerosis;
(xii) diabetes;
(xiii) acromegaly, gigantism and associated disorders;
(xiv) excessively lean body mass or a deficiency in body fat content.

39. Use of the animal model according to any one of claims 1 to 11 for the study of diseases or symptoms associated with growth hormone activity deficiency or IGF-1 deficiency such as growth retardation or deficiency, in particular dwarfism, Laron Syndrome, Illig-type deficiency, and the Kowarsky syndrome, pituitary gland deficiency, reduced bone mineral density, increased body fat accumulation, osteoporosis, information processing and cognitive function defects, age-related memory and behaviour deficits, glucose metabolism disorders and cardiovascular disorders.

40. Use of the animal model according to any one of claims 1 to 11 for the identification of early diagnostic markers for diseases associated with growth hormone activity deficiency or IGF-1 deficiency such as growth retardation or deficiency, in particular dwarfism, Laron Syndrome, Illig-type deficiency, and the Kowarsky syndrome, pituitary gland deficiency, reduced bone mineral density, increased body fat accumulation, osteoporosis, information processing and cognitive function defects, age-related memory and behaviour deficits, glucose metabolism disorders and cardiovascular disorders.

41. Use of the animal model according to any one of claims 1 to 11 for the monitoring of the activity of agents useful in the prevention or treatment of diseases or symptoms associated with growth retardation or deficiency, in particular dwarfism, Laron Syndrome, Illig-type deficiency, and the Kowarsky syndrome, pituitary gland deficiency, reduced bone mineral density, increased body fat accumulation, osteoporosis, information processing and cognitive function defects, age-related memory and behaviour deficits, glucose metabolism disorders and cardiovascular disorders.

42. Use of the animal model according to any one of claims 1 to 11 as test model system for agents suspected of promoting or aggravating diseases associated with growth retardation or deficiency, in particular dwarfism, Laron Syndrome, Illig-type deficiency, and the

Kowarsky syndrome, pituitary gland deficiency, reduced bone mineral density, increased body fat accumulation, osteoporosis, information processing and cognitive function defects, age-related memory and behaviour deficits, glucose metabolism disorders and cardiovascular disorders.

43. Use of the animal model according to any one of claims 1 to 11 for the dissection of the molecular mechanisms of the growth hormone activity pathway.

44. Use of the animal model according to any one of claims 1 to 11 for the identification and cloning of modifier genes able to modify, aggravate, reduce or inhibit the phenotype associated with a growth hormone activity deficiency.

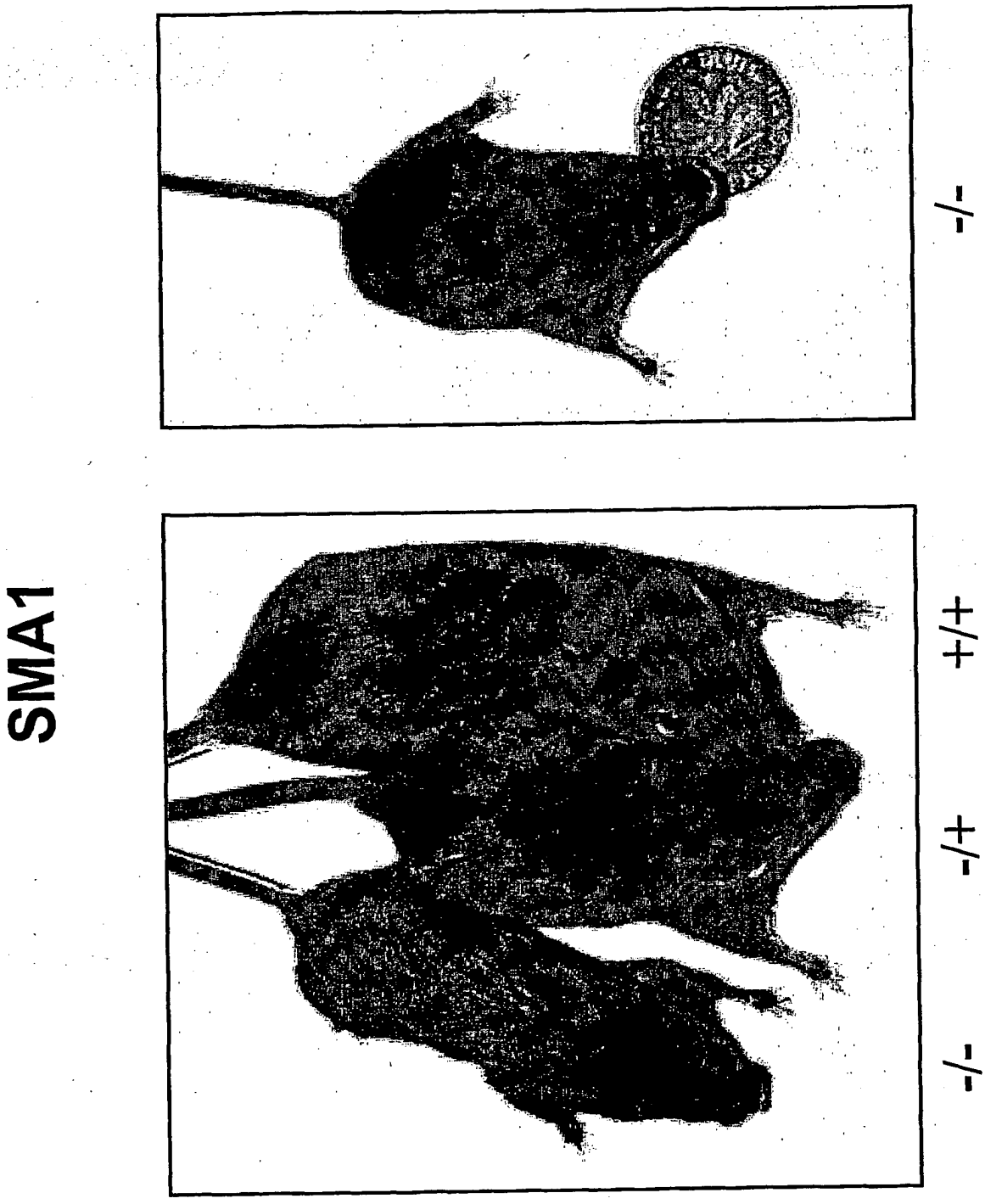
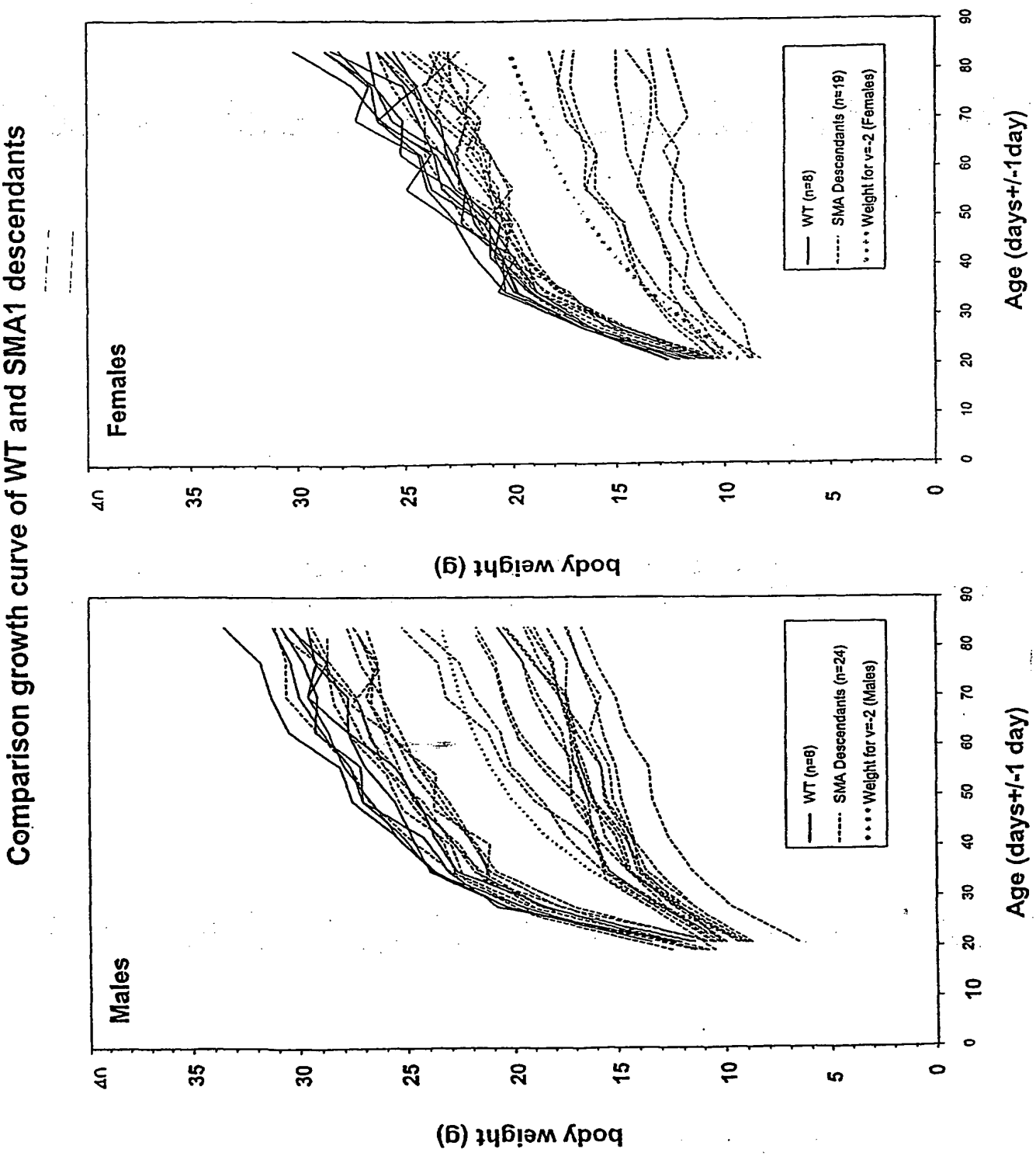


Fig. 1



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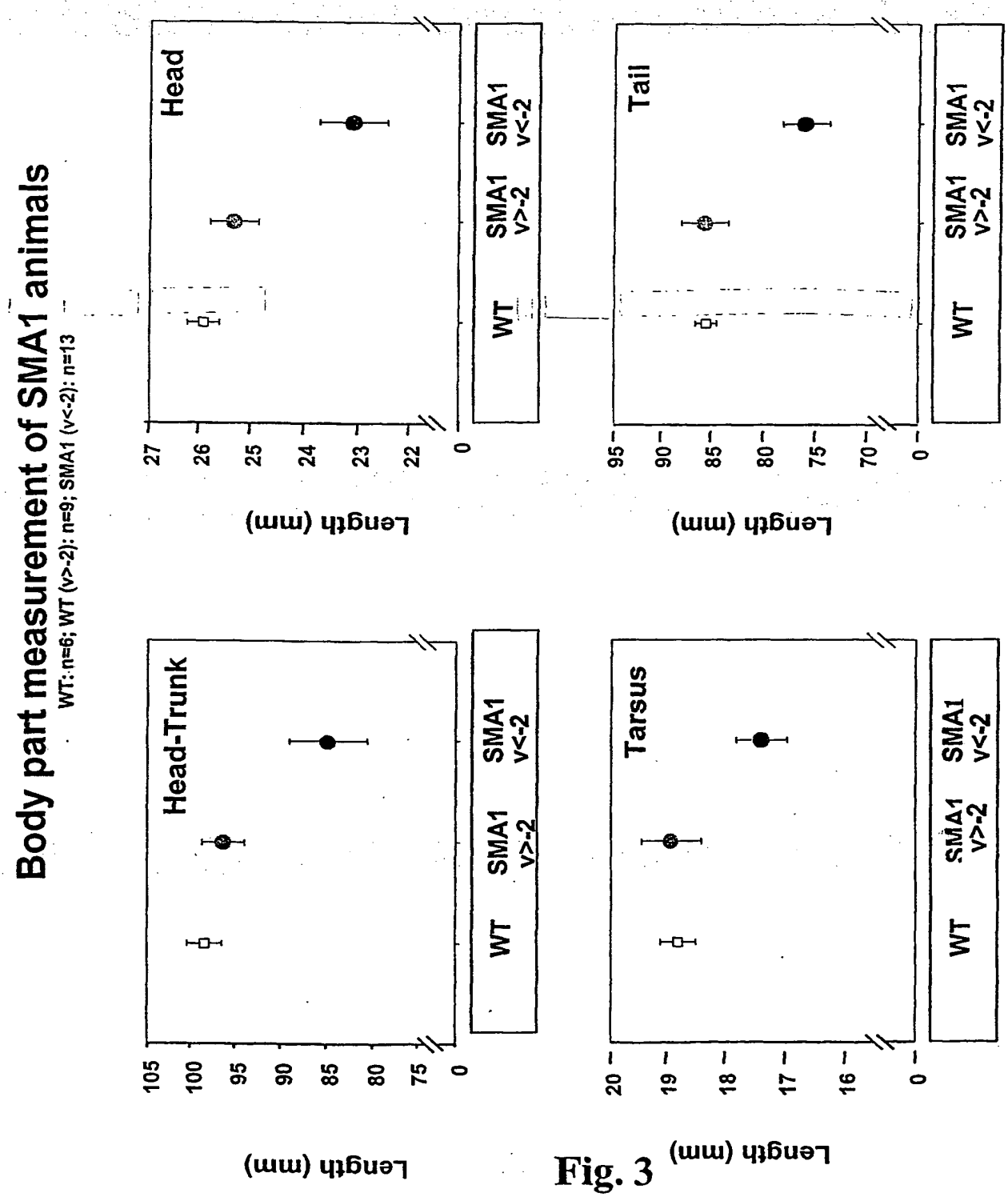


Fig. 3

Body mass and naso-anal length of WT and SMA1 mice

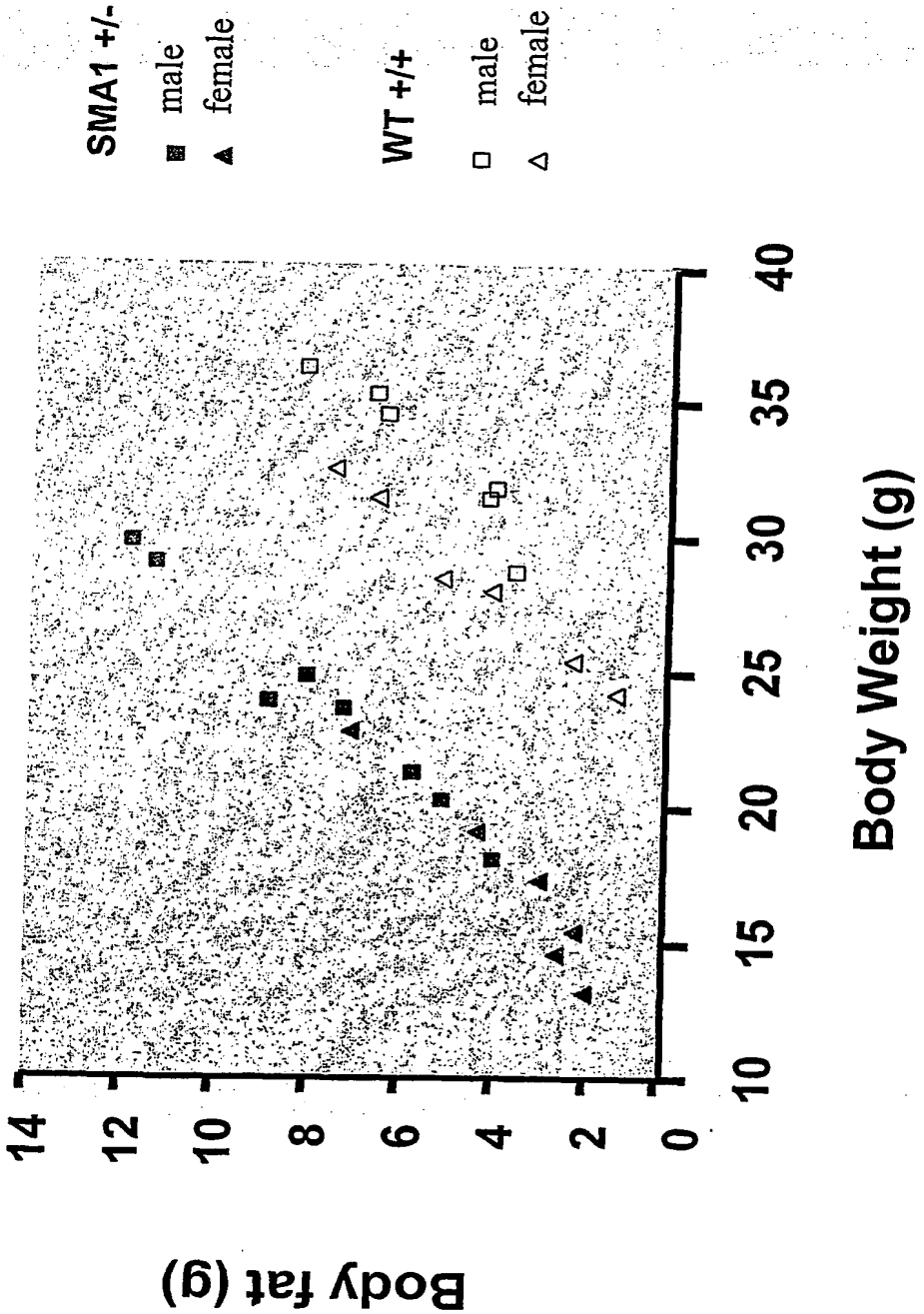
(at 8 weeks of age)

males	body weight (g)		length (mm)		n
WT	25.5 (+/- 1.1)	100%	96 (+/- 2)	100%	9
Heterozygotes SMA-1	16.9 (+/- 1.9)	66%	85 (+/- 4)	88%	13
Homozygotes SMA-1	11.1 (+/- 0.1)	43%	n.d	-	2

females	body weight (g)		length (mm)		n
WT	21.4 (+/- 1.0)	100 %	94 (+/- 2)	100%	12
Heterozygotes SMA-1	14.2 (+/- 1.9)	66%	80 (+/- 3)	85%	6
Homozygotes SMA-1	9.7/9.3	44%	70/ 72	75 %	2

- Numbers in ( ) indicate mean ±SE
-n indicates the number of animals examined
-The % is calculated in comparision to the WT value

Fig. 4



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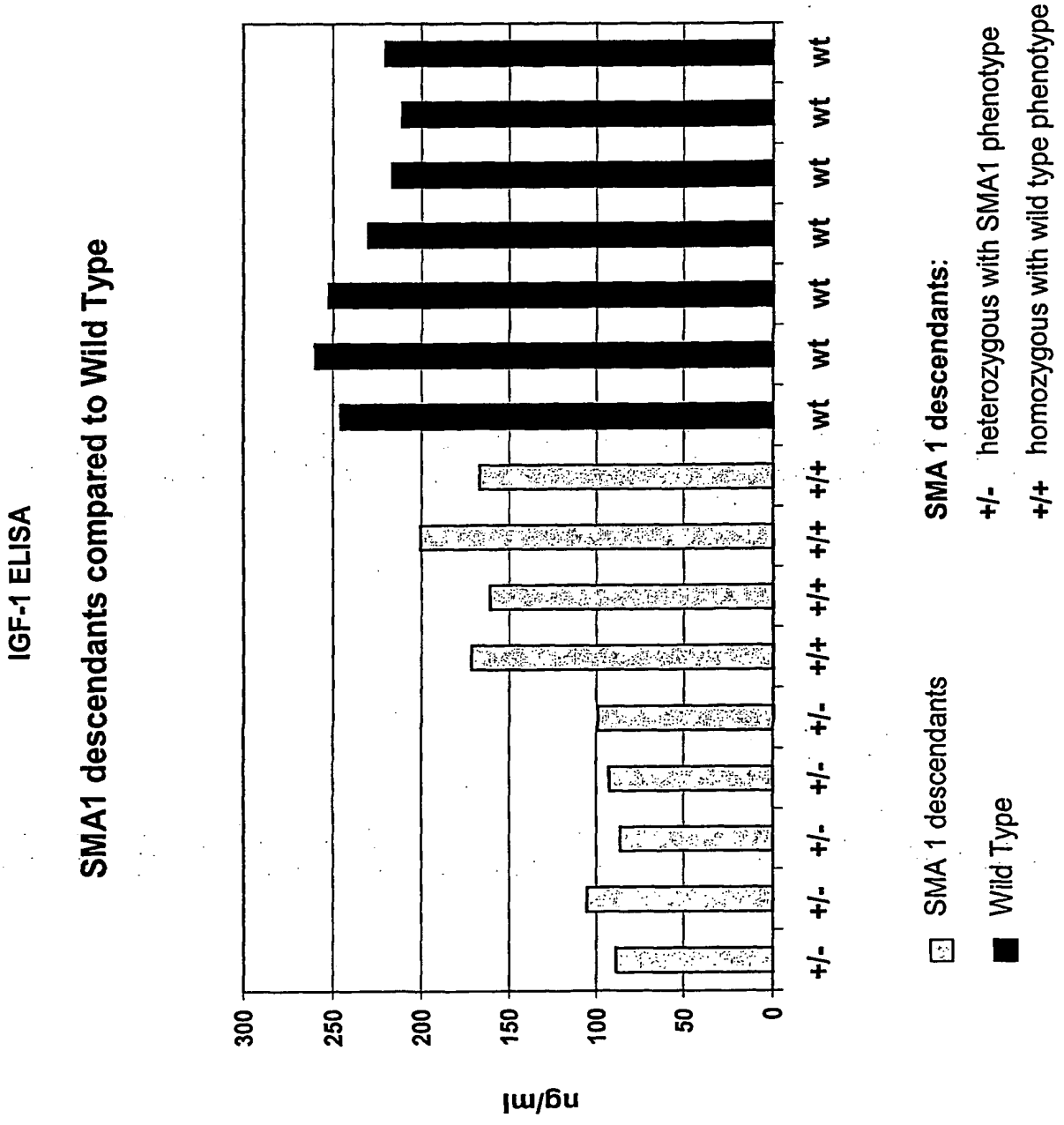

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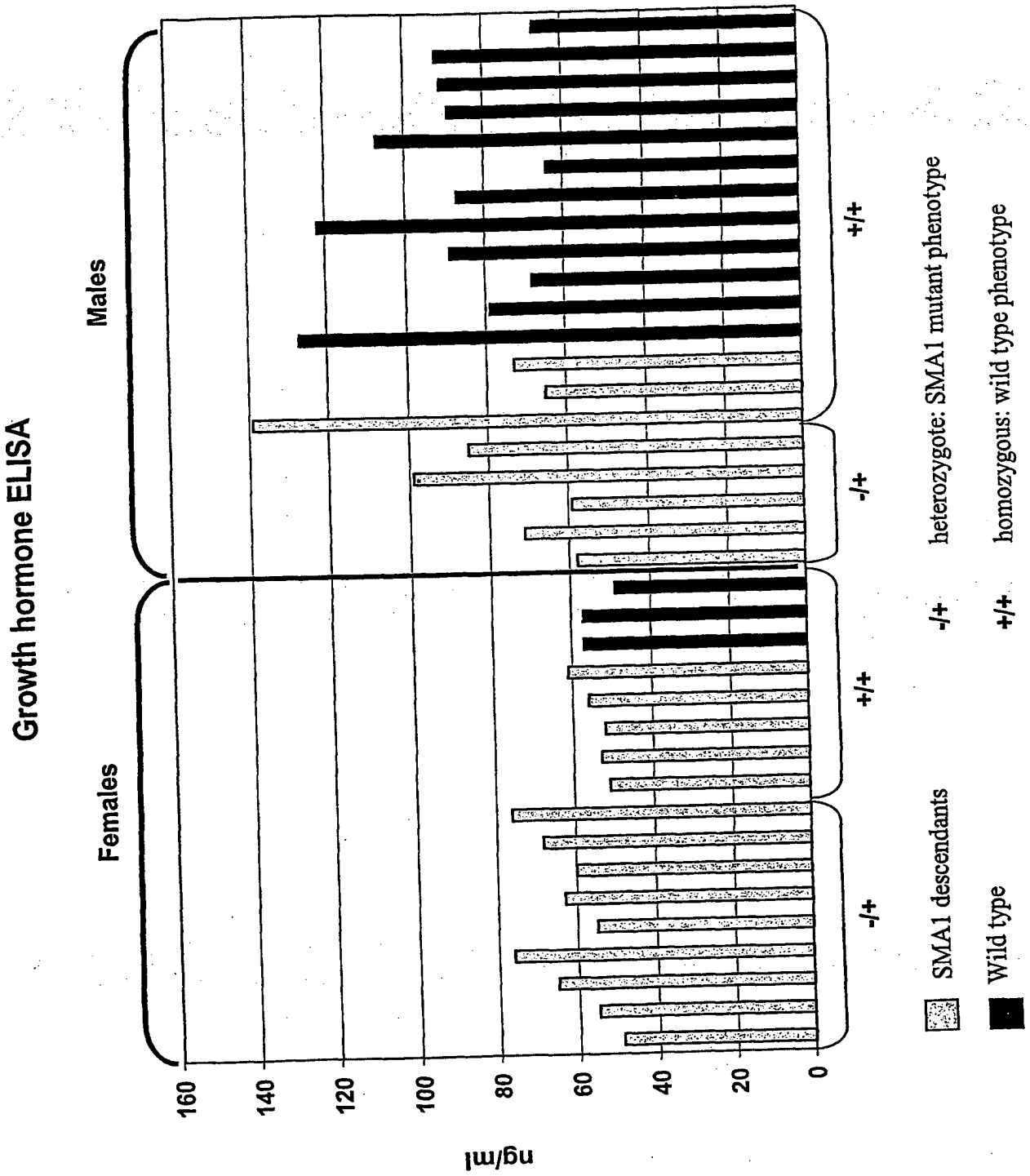
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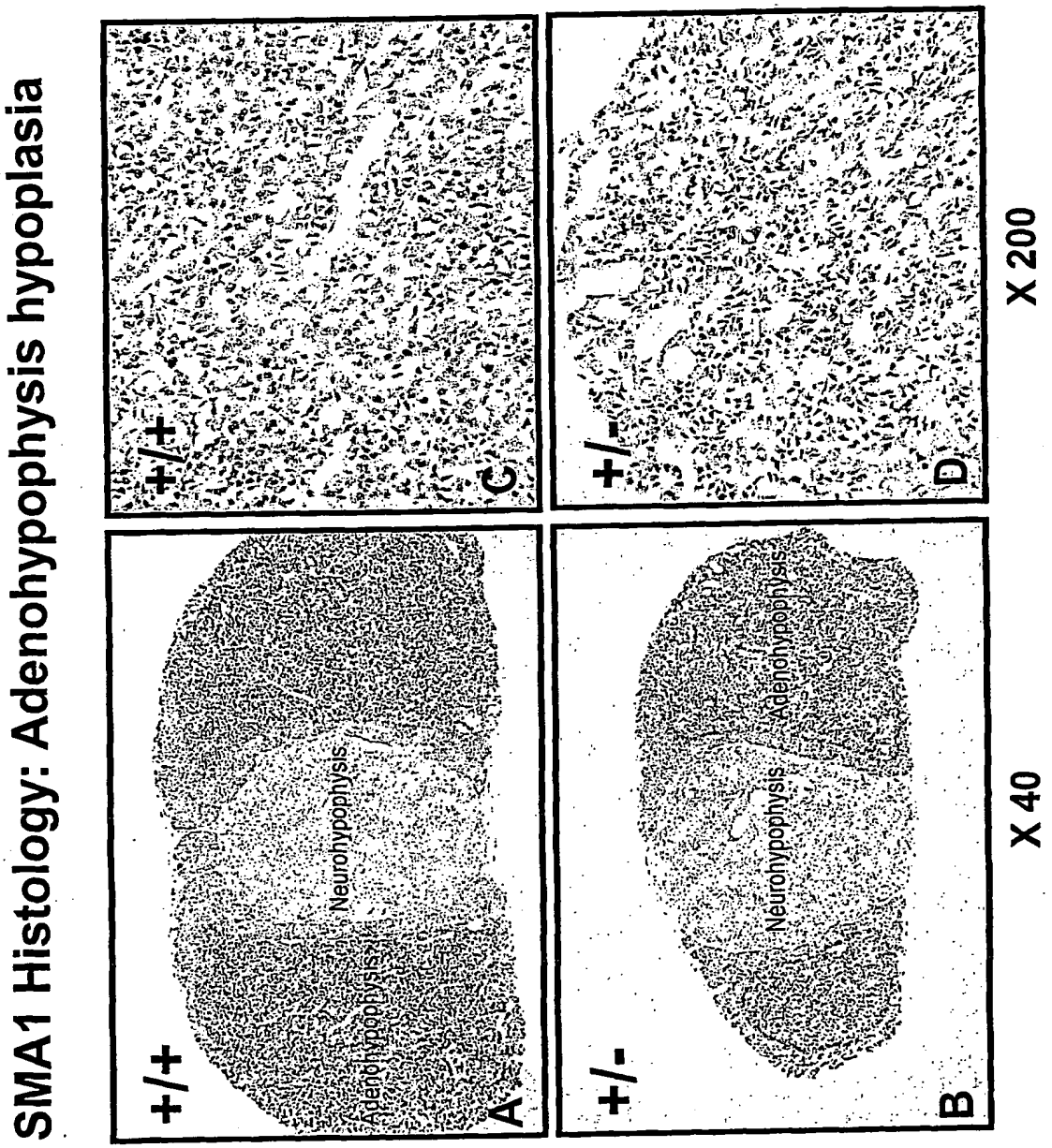
Females
Males
-/+
+/+
-/+
+/+
ng/ml
0
20
40
60
80
100
120
140
160
SMA1 descendants
Wild type
-/+ heterozygote: SMA1 mutant phenotype
+/+ homozygous: wild type phenotype

Fig. 7



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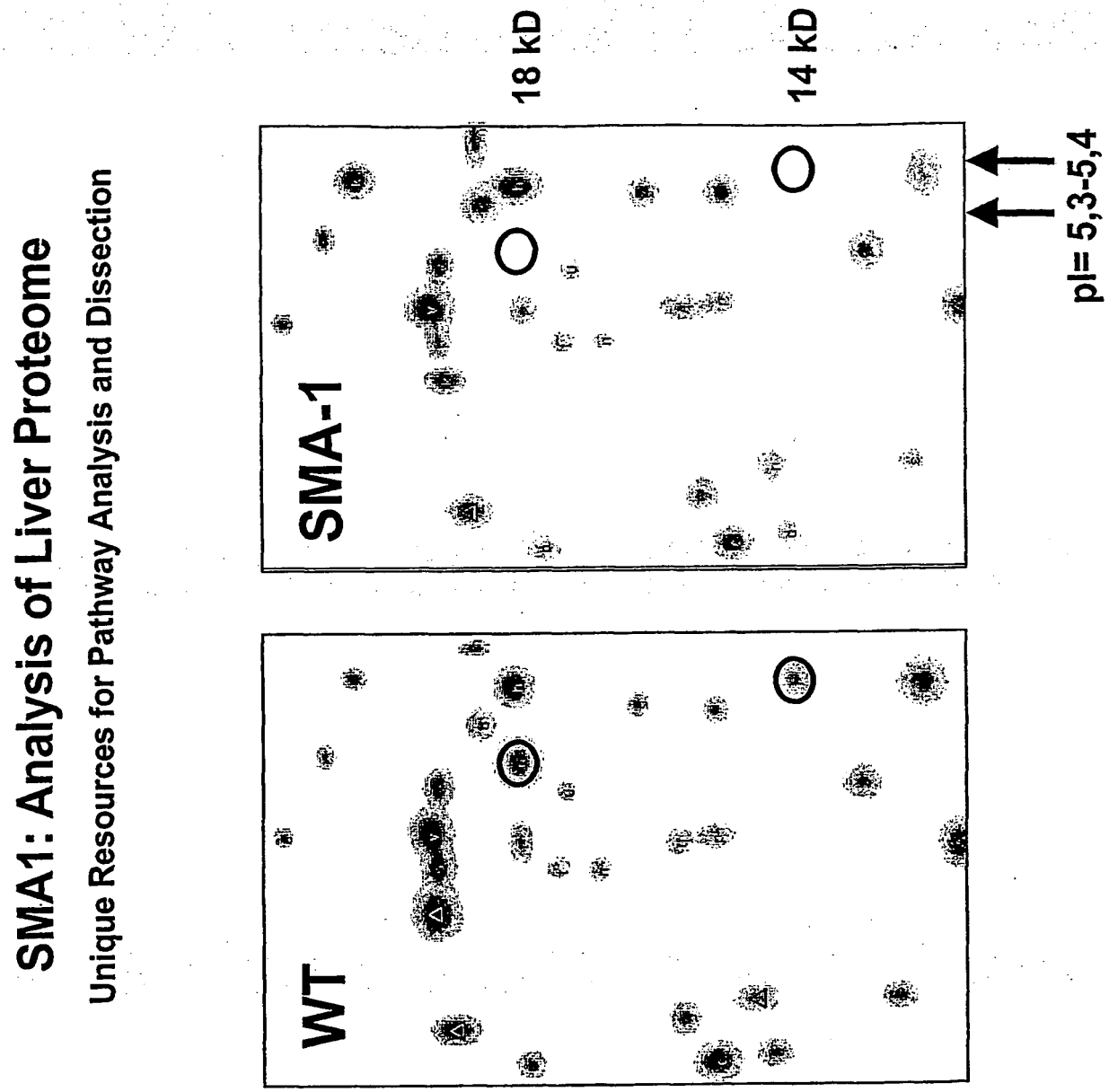
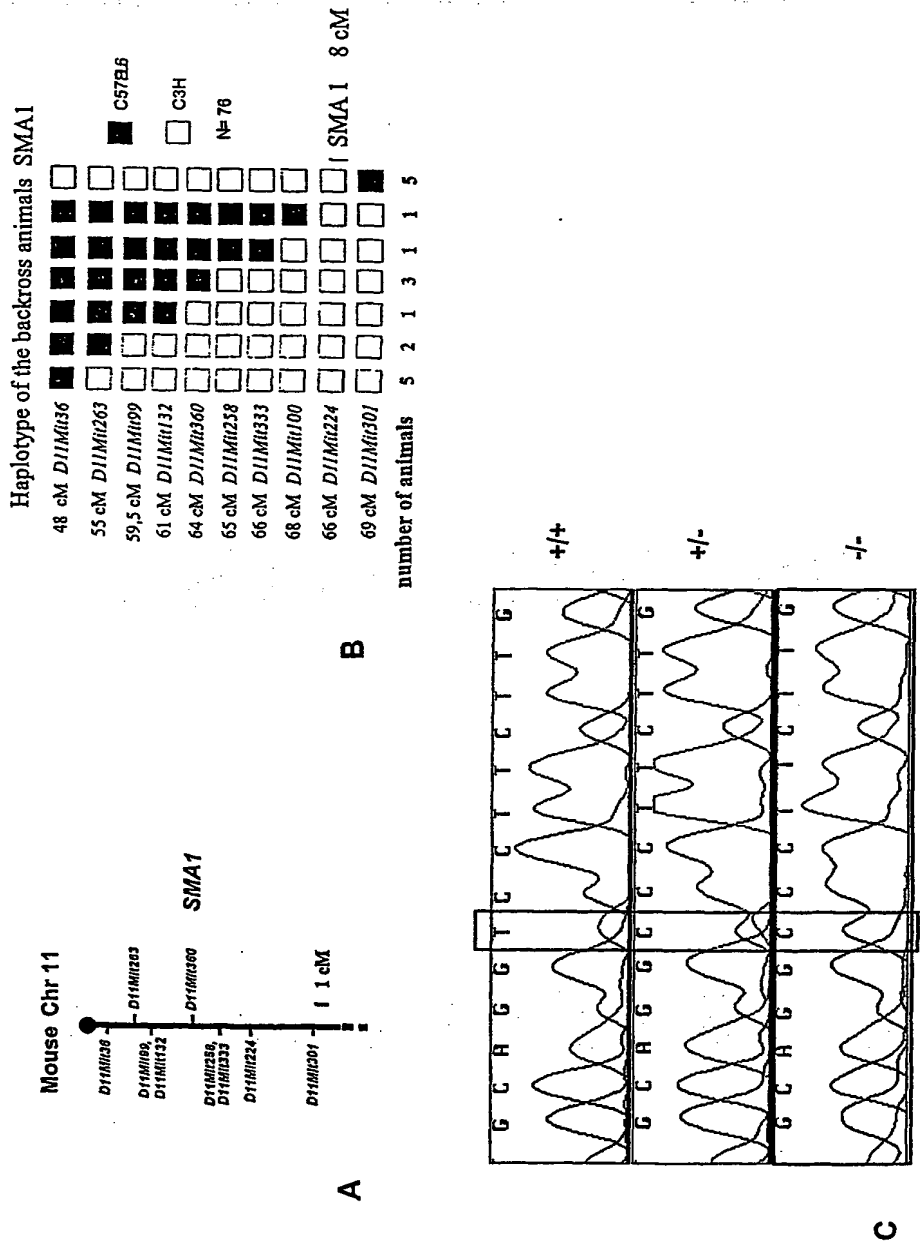


Fig. 9

Differentially expressed proteins in the liver of SMA-1 and wild type mice
pH 4.5-5.5

Spot Nr.	Protein Name	Accession	Mass in Dalton	Regulation in SMA1 versus WT
1	Glutathione S-transferase (mouse)	GTP1 MOUSE	23634	**downregulated**
2	Myosin light chain 1 (mouse)	MLE1 MOUSE	20564	**downregulated**
3	Major urinary protein 1 (MUP) (mouse)	MUP1 MOUSE	20920	**downregulated**
4	Major urinary protein 2 (MUP) (mouse)	MUP2 MOUSE	20935	**downregulated**

Fig. 10



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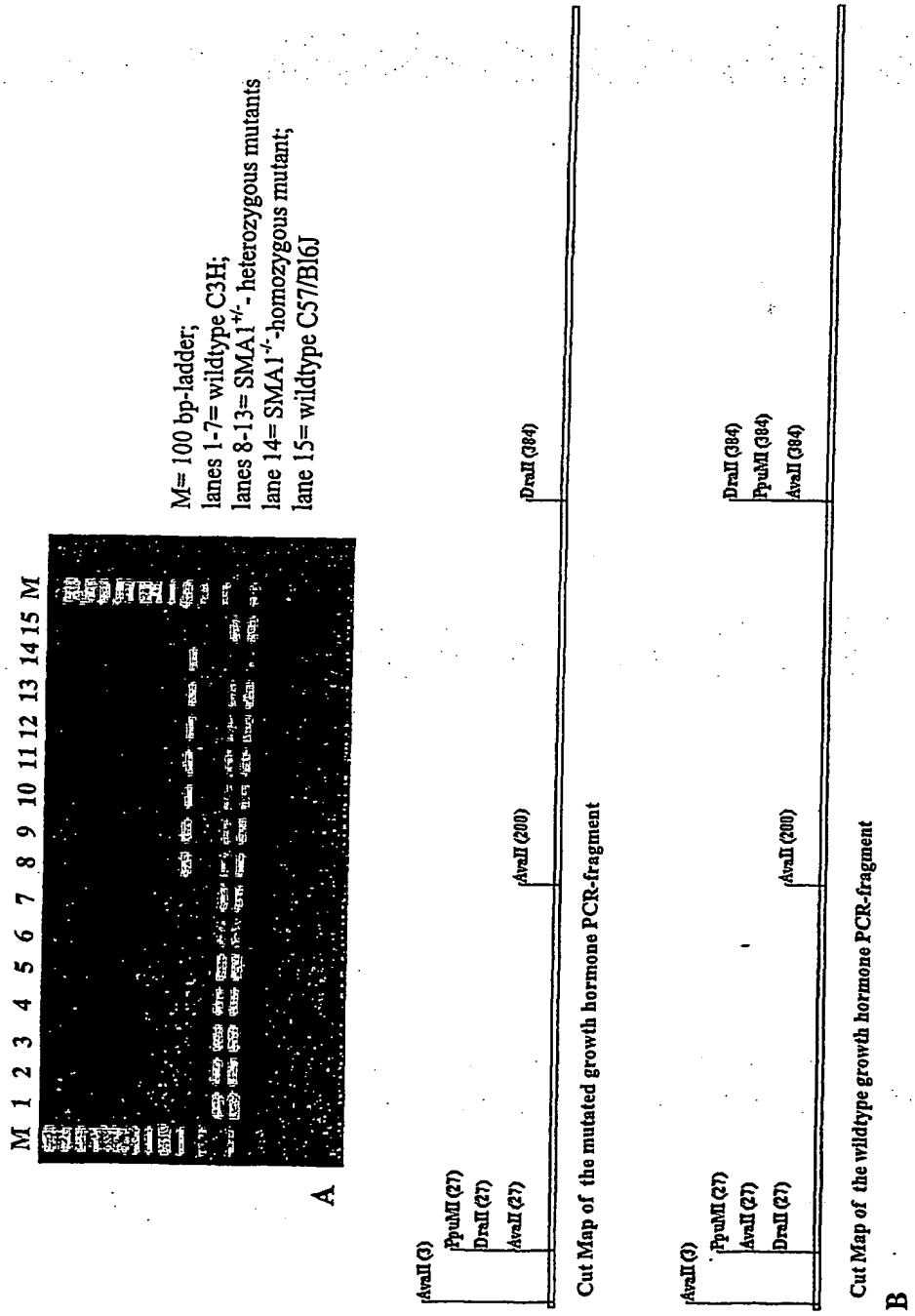

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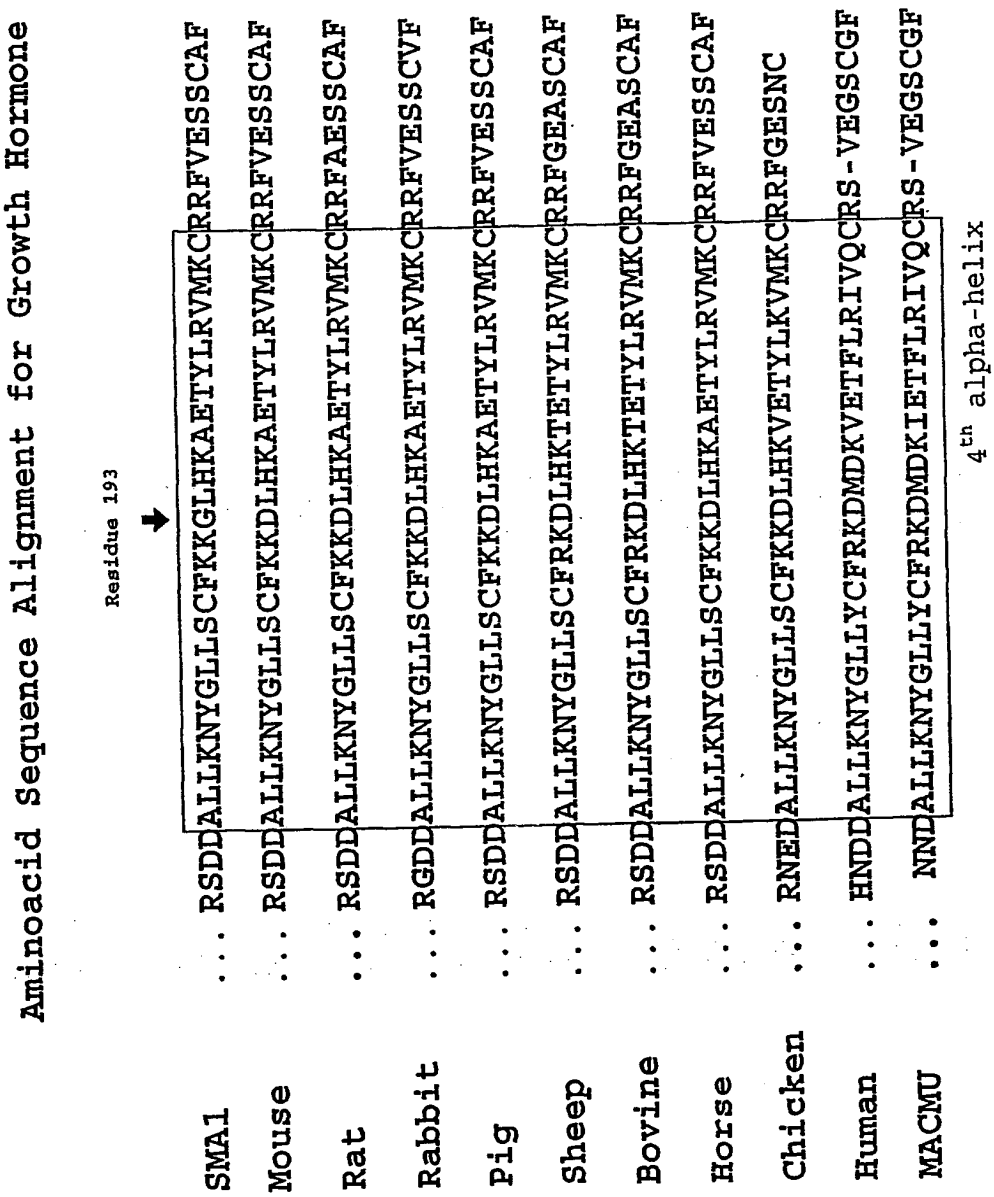
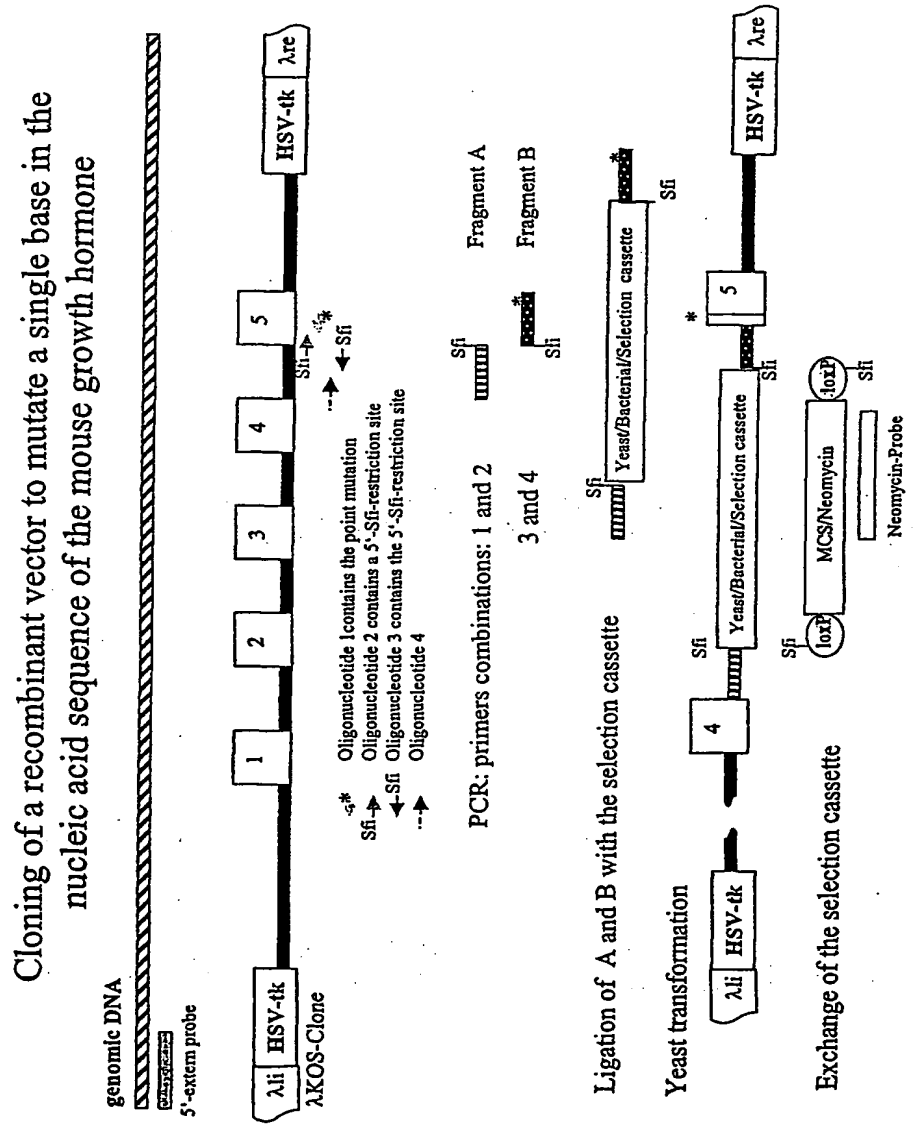


Fig. 13



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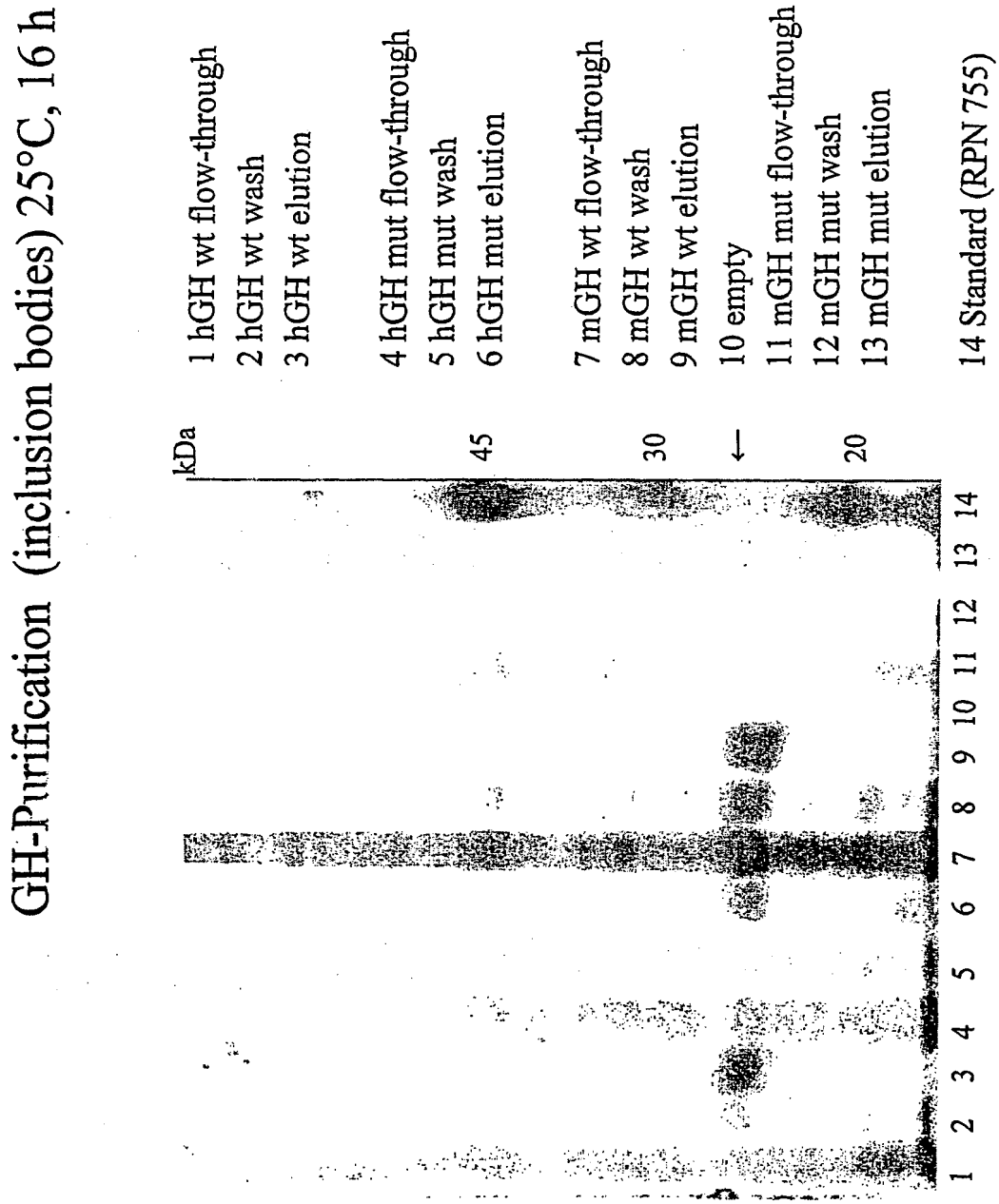

Fig. 15

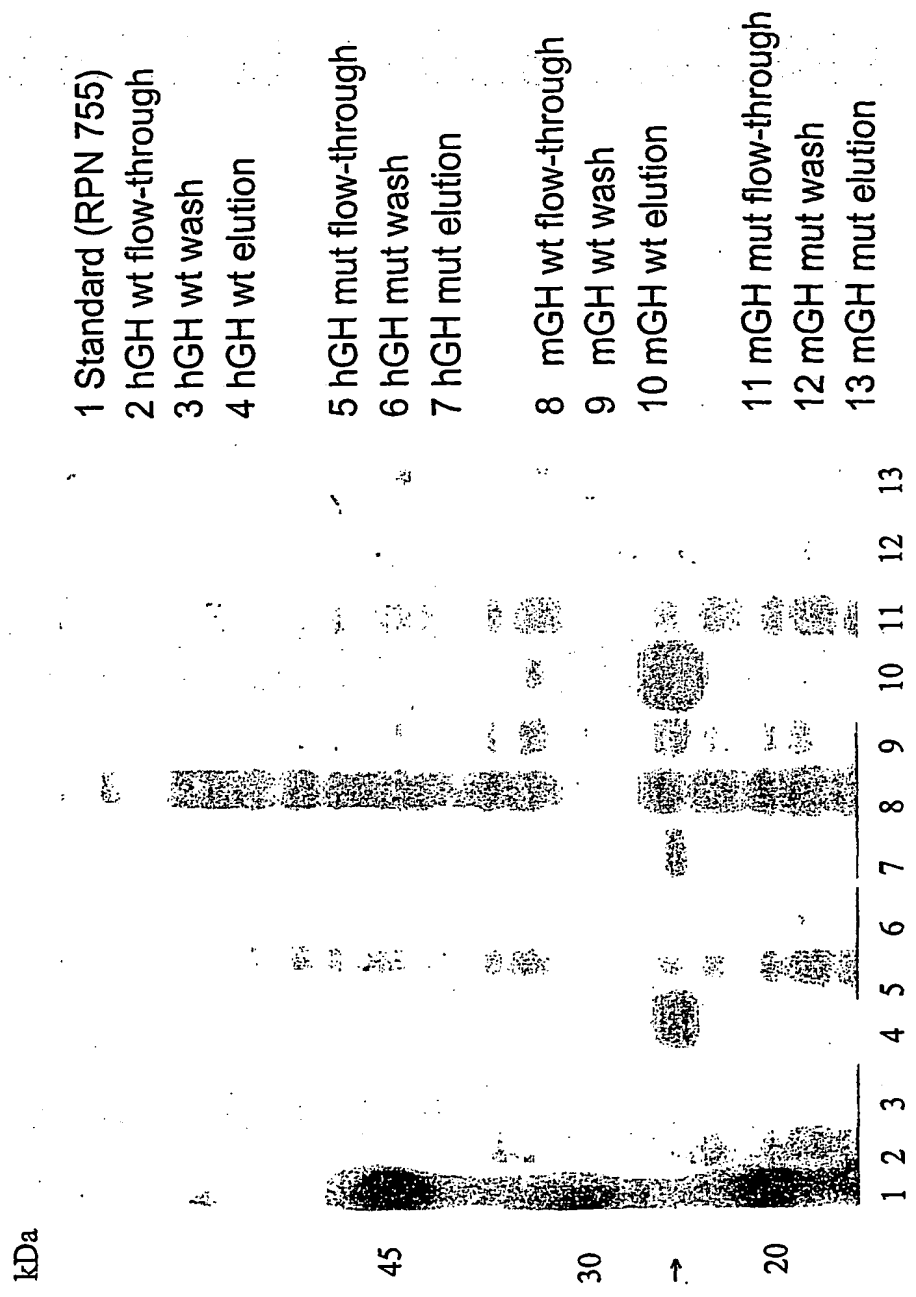

Fig. 16

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                 85                  90                  95
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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 19
ggatgaaggc acagctgctt tcc 23

<210> 20
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 20
tcatcatgcc gtctgtgatg 20

<210> 21
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 21
gcagcgcatc gccttctatc 20

<210> 22
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 22
gaggatggac tagcctggg 19

<210> 23
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 23
cctgtcgtgg gaaagaaggg 20

<210> 24
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 24
gacaaggctg gtgggcac 18

<210> 25
<211> 18
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 25
ccaaggccca actccccg 18

<210> 26
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 26
ggcaacttcc aaggccagg 19

<210> 27
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 27
ccttgtccat gcccttcctg 20

<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 28
caggaagggc atggacaagg 20

<210> 29
<211> 769
<212> DNA
<213> Human

<220>
<221> CDS
<222> (56)..(706)

<220>
<221> sig_peptide
<222> (56)..(133)

<400> 29

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ccaaggccca actccccgaa ccactcaggg tcctgtggac gctcacctag ctgca atg  58
                                                            Met
                                                             1

gct aca ggc tcc cgg acg tcc ctg ctc ctg gct ttt ggc ctg ctc tgc  106
Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys
                5                   10                  15

ctg ccc tgg ctt caa gag ggc agt gcc ttc cca acc att ccc tta tcc  154
Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu Ser
            20                  25                  30

agg ctt ttt gac aac gct atg ctc cgc gcc cat cgt ctg cac cag ctg  202
Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu
        35                  40                  45

gcc ttt gac acc tac cag gag ttt gaa gaa gcc tat atc cca aag gaa  250
Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu
    50                  55                  60                  65

cag aag tat tca ttc ctg cag aac ccc cag acc tcc ctc tgt ttc tca  298
Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser
                70                  75                  80

gag tct att ccg aca ccc tcc aac agg gag gaa aca caa cag aaa tcc  346
Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser
            85                  90                  95

aac cta gag ctg ctc cgc atc tcc ctg ctg ctc atc cag tcg tgg ctg  394
Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu
        100                 105                 110

gag ccc gtg cag ttc ctc agg agt gtc ttc gcc aac agc ctg gtg tac  442
Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr
    115                 120                 125

ggc gcc tct gac agc aac gtc tat gac ctc cta aag gac cta gag gaa  490
Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu
130                 135                 140                 145

ggc atc caa acg ctg atg ggg agg ctg gaa gat ggc agc ccc cgg act  538
Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr
                150                 155                 160

ggg cag atc ttc aag cag acc tac agc aag ttc gac aca aac tca cac  586
Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser His
            165                 170                 175

aac gat gac gca cta ctc aag aac tac ggg ctg ctc tac tgc ttc agg  634
Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg
        180                 185                 190

aag gac atg gac aag gtc gag aca ttc ctg cgc atc gtg cag tgc cgc  682
Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg
    195                 200                 205

tct gtg gag ggc agc tgt ggc ttc tagctgcccg ggtggcatcc ctgtgacccc  736
Ser Val Glu Gly Ser Cys Gly Phe
210                 215

tccccagtgc ctctcctggc cttggaagtt gcc                              769
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<210> 30
<211> 217
<212> PRT
<213> Human

<400> 30
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1 5 10 15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
20 25 30

Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
35 40 45

Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
50 55 60

Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65 70 75 80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
85 90 95

Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
100 105 110

Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
115 120 125

Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
130 135 140

Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
145 150 155 160

Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
165 170 175

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
180 185 190

Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
195 200 205

Arg Ser Val Glu Gly Ser Cys Gly Phe
210 215

<210> 31
<211> 769
<212> DNA
<213> Human

<220>
<221> CDS
<222> (56)..(706)

<220>
<221> sig_peptide
<222> (56)..(133)

<220>
<221> mutation
<222> (639)
<223> A to G transition

<400> 31

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ccaaggccca actccccgaa ccactcaggg tcctgtggac gctcacctag ctgca atg  58
                                                           Met
                                                             1

gct aca ggc tcc cgg acg tcc ctg ctc ctg gct ttt ggc ctg ctc tgc  106
Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys
              5                  10                  15

ctg ccc tgg ctt caa gag ggc agt gcc ttc cca acc att ccc tta tcc  154
Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu Ser
          20                  25                  30

agg ctt ttt gac aac gct atg ctc cgc gcc cat cgt ctg cac cag ctg  202
Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu
      35                  40                  45

gcc ttt gac acc tac cag gag ttt gaa gaa gcc tat atc cca aag gaa  250
Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu
  50                  55                  60                  65

cag aag tat tca ttc ctg cag aac ccc cag acc tcc ctc tgt ttc tca  298
Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser
                  70                  75                  80

gag tct att ccg aca ccc tcc aac agg gag gaa aca caa cag aaa tcc  346
Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser
              85                  90                  95

aac cta gag ctg ctc cgc atc tcc ctg ctg ctc atc cag tcg tgg ctg  394
Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu
          100                 105                 110

gag ccc gtg cag ttc ctc agg agt gtc ttc gcc aac agc ctg gtg tac  442
Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr
      115                 120                 125

ggc gcc tct gac agc aac gtc tat gac ctc cta aag gac cta gag gaa  490
Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu
130                 135                 140                 145

ggc atc caa acg ctg atg ggg agg ctg gaa gat ggc agc ccc cgg act  538
Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr
                  150                 155                 160

ggg cag atc ttc aag cag acc tac agc aag ttc gac aca aac tca cac  586
Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser His
              165                 170                 175

aac gat gac gca cta ctc aag aac tac ggg ctg ctc tac tgc ttc agg  634
Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg
          180                 185                 190

aag ggc atg gac aag gtc gag aca ttc ctg cgc atc gtg cag tgc cgc  682
Lys Gly Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg
      195                 200                 205

tct gtg gag ggc agc tgt ggc ttc tagctgcccg ggtggcatcc ctgtgacccc  736
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Ser Val Glu Gly Ser Cys Gly Phe
210 215

tccccagtgc ctctcctggc cttggaagtt gcc 769

<210> 32
<211> 217
<212> PRT
<213> Human

<400> 32
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1 5 10 15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
20 25 30

Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
35 40 45

Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
50 55 60

Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65 70 75 80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
85 90 95

Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
100 105 110

Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
115 120 125

Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
130 135 140

Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
145 150 155 160

Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
165 170 175

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
180 185 190

Arg Lys Gly Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
195 200 205

Arg Ser Val Glu Gly Ser Cys Gly Phe
210 215

<210> 33
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:

Oligonucleotide

<400> 33
ggtggcagtt gccagggg 18

<210> 34
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 34
gagtccagat tccaaactgc 20

<210> 35
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 35
gacactggtg agtggctag 19

<210> 36
<211> 718
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (53)..(700)

<220>
<221> sig_peptide
<222> (53)..(130)

<400> 36
gagtccagat tccaaactgc tcagagtcct gtggacagat cactgcttgg ca atg gct 58
Met Ala
1

aca gac tct cgg acc tcc tgg ctc ctg acc gtc agc ctg ctc tgc ctg 106
Thr Asp Ser Arg Thr Ser Trp Leu Leu Thr Val Ser Leu Leu Cys Leu
5 10 15

ctc tgg cct cag gag gct agt gct ttt ccc gcc atg ccc ttg tcc agt 154
Leu Trp Pro Gln Glu Ala Ser Ala Phe Pro Ala Met Pro Leu Ser Ser
20 25 30

ctg ttt tct aat gct gtg ctc cga gcc cag cac ctg cac cag ctg gct 202
Leu Phe Ser Asn Ala Val Leu Arg Ala Gln His Leu His Gln Leu Ala
35 40 45 50

gct gac acc tac aaa gag ttc gag cgt gcc tac att ccc gag gga cag 250

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Ala Asp Thr Tyr Lys Glu Phe Glu Arg Ala Tyr Ile Pro Glu Gly Gln
                55                  60                  65

cgc tat tcc att cag aat gcc cag gct gct ttc tgc ttc tca gag acc   298
Arg Tyr Ser Ile Gln Asn Ala Gln Ala Ala Phe Cys Phe Ser Glu Thr
            70                  75                  80

atc ccg gcc ccc aca ggc aag gag gag gcc cag cag aga acc gac atg   346
Ile Pro Ala Pro Thr Gly Lys Glu Glu Ala Gln Gln Arg Thr Asp Met
        85                  90                  95

gaa ttg ctt cgc ttc tcg ctg ctg ctc atc cag tca tgg ctg ggg ccc   394
Glu Leu Leu Arg Phe Ser Leu Leu Leu Ile Gln Ser Trp Leu Gly Pro
    100                 105                 110

gtg cag ttc ctc agc agg att ttc acc aac agc ctg atg ttc ggc acc   442
Val Gln Phe Leu Ser Arg Ile Phe Thr Asn Ser Leu Met Phe Gly Thr
115                 120                 125                 130

tcg gac cgt gtc tat gag aaa ctg aag gac ctg gaa gag ggc atc cag   490
Ser Asp Arg Val Tyr Glu Lys Leu Lys Asp Leu Glu Glu Gly Ile Gln
                135                 140                 145

gct ctg atg cag gag ctg gaa gat ggc agc ccc cgt gtt ggg cag atc   538
Ala Leu Met Gln Glu Leu Glu Asp Gly Ser Pro Arg Val Gly Gln Ile
            150                 155                 160

ctc aag caa acc tat gac aag ttt gac gcc aac atg cgc agc gac gac   586
Leu Lys Gln Thr Tyr Asp Lys Phe Asp Ala Asn Met Arg Ser Asp Asp
        165                 170                 175

gcg ctg ctc aaa aac tat ggg ctg ctc tcc tgc ttc aag aag gac ctg   634
Ala Leu Leu Lys Asn Tyr Gly Leu Leu Ser Cys Phe Lys Lys Asp Leu
    180                 185                 190

cac aaa gcg gag acc tac ctg cgg gtc atg aag tgt cgc cgc ttt gtg   682
His Lys Ala Glu Thr Tyr Leu Arg Val Met Lys Cys Arg Arg Phe Val
195                 200                 205                 210

gaa agc agc tgt gcc ttc tagccactca ccagtgtc                      718
Glu Ser Ser Cys Ala Phe
                215
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<210> 37
<211> 216
<212> PRT
<213> Mus musculus

<400> 37

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Met Ala Thr Asp Ser Arg Thr Ser Trp Leu Leu Thr Val Ser Leu Leu
  1               5                  10                  15

Cys Leu Leu Trp Pro Gln Glu Ala Ser Ala Phe Pro Ala Met Pro Leu
            20                  25                  30

Ser Ser Leu Phe Ser Asn Ala Val Leu Arg Ala Gln His Leu His Gln
        35                  40                  45

Leu Ala Ala Asp Thr Tyr Lys Glu Phe Glu Arg Ala Tyr Ile Pro Glu
    50                  55                  60

Gly Gln Arg Tyr Ser Ile Gln Asn Ala Gln Ala Ala Phe Cys Phe Ser
```

65 70 75 80

Glu Thr Ile Pro Ala Pro Thr Gly Lys Glu Glu Ala Gln Gln Arg Thr
85 90 95

Asp Met Glu Leu Leu Arg Phe Ser Leu Leu Leu Ile Gln Ser Trp Leu
100 105 110

Gly Pro Val Gln Phe Leu Ser Arg Ile Phe Thr Asn Ser Leu Met Phe
115 120 125

Gly Thr Ser Asp Arg Val Tyr Glu Lys Leu Lys Asp Leu Glu Glu Gly
130 135 140

Ile Gln Ala Leu Met Gln Glu Leu Glu Asp Gly Ser Pro Arg Val Gly
145 150 155 160

Gln Ile Leu Lys Gln Thr Tyr Asp Lys Phe Asp Ala Asn Met Arg Ser
165 170 175

Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Ser Cys Phe Lys Lys
180 185 190

Asp Leu His Lys Ala Glu Thr Tyr Leu Arg Val Met Lys Cys Arg Arg
195 200 205

Phe Val Glu Ser Ser Cys Ala Phe
210 215

<210> 38
<211> 718
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (53)..(700)

<220>
<221> sig_peptide
<222> (53)..(130)

<220>
<221> mutation
<222> (630)
<223> A to G transition

<400> 38
gagtccagat tccaaactgc tcagagtcct gtggacagat cactgcttgg ca atg gct 58
Met Ala
1

aca gac tct cgg acc tcc tgg ctc ctg acc gtc agc ctg ctc tgc ctg 106
Thr Asp Ser Arg Thr Ser Trp Leu Leu Thr Val Ser Leu Leu Cys Leu
5 10 15

ctc tgg cct cag gag gct agt gct ttt ccc gcc atg ccc ttg tcc agt 154
Leu Trp Pro Gln Glu Ala Ser Ala Phe Pro Ala Met Pro Leu Ser Ser
20 25 30

ctg ttt tct aat gct gtg ctc cga gcc cag cac ctg cac cag ctg gct 202

```
Leu Phe Ser Asn Ala Val Leu Arg Ala Gln His Leu His Gln Leu Ala
 35                  40                  45                  50

gct gac acc tac aaa gag ttc gag cgt gcc tac att ccc gag gga cag   250
Ala Asp Thr Tyr Lys Glu Phe Glu Arg Ala Tyr Ile Pro Glu Gly Gln
                 55                  60                  65

cgc tat tcc att cag aat gcc cag gct gct ttc tgc ttc tca gag acc   298
Arg Tyr Ser Ile Gln Asn Ala Gln Ala Ala Phe Cys Phe Ser Glu Thr
             70                  75                  80

atc ccg gcc ccc aca ggc aag gag gag gcc cag cag aga acc gac atg   346
Ile Pro Ala Pro Thr Gly Lys Glu Glu Ala Gln Gln Arg Thr Asp Met
         85                  90                  95

gaa ttg ctt cgc ttc tcg ctg ctg ctc atc cag tca tgg ctg ggg ccc   394
Glu Leu Leu Arg Phe Ser Leu Leu Leu Ile Gln Ser Trp Leu Gly Pro
    100                 105                 110

gtg cag ttc ctc agc agg att ttc acc aac agc ctg atg ttc ggc acc   442
Val Gln Phe Leu Ser Arg Ile Phe Thr Asn Ser Leu Met Phe Gly Thr
115                 120                 125                 130

tcg gac cgt gtc tat gag aaa ctg aag gac ctg gaa gag ggc atc cag   490
Ser Asp Arg Val Tyr Glu Lys Leu Lys Asp Leu Glu Glu Gly Ile Gln
                135                 140                 145

gct ctg atg cag gag ctg gaa gat ggc agc ccc cgt gtt ggg cag atc   538
Ala Leu Met Gln Glu Leu Glu Asp Gly Ser Pro Arg Val Gly Gln Ile
            150                 155                 160

ctc aag caa acc tat gac aag ttt gac gcc aac atg cgc agc gac gac   586
Leu Lys Gln Thr Tyr Asp Lys Phe Asp Ala Asn Met Arg Ser Asp Asp
        165                 170                 175

gcg ctg ctc aaa aac tat ggg ctg ctc tcc tgc ttc aag aag ggc ctg   634
Ala Leu Leu Lys Asn Tyr Gly Leu Leu Ser Cys Phe Lys Lys Gly Leu
    180                 185                 190

cac aaa gcg gag acc tac ctg cgg gtc atg aag tgt cgc cgc ttt gtg   682
His Lys Ala Glu Thr Tyr Leu Arg Val Met Lys Cys Arg Arg Phe Val
195                 200                 205                 210

gaa agc agc tgt gcc ttc tagccactca ccagtgtc                      718
Glu Ser Ser Cys Ala Phe
                215
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<210> 39
<211> 216
<212> PRT
<213> Mus musculus

<400> 39

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Met Ala Thr Asp Ser Arg Thr Ser Trp Leu Leu Thr Val Ser Leu Leu
  1               5                  10                  15

Cys Leu Leu Trp Pro Gln Glu Ala Ser Ala Phe Pro Ala Met Pro Leu
             20                  25                  30

Ser Ser Leu Phe Ser Asn Ala Val Leu Arg Ala Gln His Leu His Gln
         35                  40                  45
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Leu Ala Ala Asp Thr Tyr Lys Glu Phe Glu Arg Ala Tyr Ile Pro Glu
     50                  55                  60

Gly Gln Arg Tyr Ser Ile Gln Asn Ala Gln Ala Ala Phe Cys Phe Ser
 65                  70                  75                  80

Glu Thr Ile Pro Ala Pro Thr Gly Lys Glu Glu Ala Gln Gln Arg Thr
                85                  90                  95

Asp Met Glu Leu Leu Arg Phe Ser Leu Leu Leu Ile Gln Ser Trp Leu
            100                 105                 110

Gly Pro Val Gln Phe Leu Ser Arg Ile Phe Thr Asn Ser Leu Met Phe
        115                 120                 125

Gly Thr Ser Asp Arg Val Tyr Glu Lys Leu Lys Asp Leu Glu Glu Gly
    130                 135                 140

Ile Gln Ala Leu Met Gln Glu Leu Glu Asp Gly Ser Pro Arg Val Gly
145                 150                 155                 160

Gln Ile Leu Lys Gln Thr Tyr Asp Lys Phe Asp Ala Asn Met Arg Ser
                165                 170                 175

Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Ser Cys Phe Lys Lys
            180                 185                 190

Gly Leu His Lys Ala Glu Thr Tyr Leu Arg Val Met Lys Cys Arg Arg
        195                 200                 205

Phe Val Glu Ser Ser Cys Ala Phe
    210                 215
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<210> 40
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 40
gtacaagctt tttcccgcca tgcccttg 28

<210> 41
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 41
catgaagctt ctagaaggca cagctgc 27

<210> 42
<211> 573

<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(570)
<223> no signal peptide sequence

<400> 42

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ttt ccc gcc atg ccc ttg tcc agt ctg ttt tct aat gct gtg ctc cga   48
Phe Pro Ala Met Pro Leu Ser Ser Leu Phe Ser Asn Ala Val Leu Arg
  1               5                  10                  15

gcc cag cac ctg cac cag ctg gct gct gac acc tac aaa gag ttc gag   96
Ala Gln His Leu His Gln Leu Ala Ala Asp Thr Tyr Lys Glu Phe Glu
             20                  25                  30

cgt gcc tac att ccc gag gga cag cgc tat tcc att cag aat gcc cag  144
Arg Ala Tyr Ile Pro Glu Gly Gln Arg Tyr Ser Ile Gln Asn Ala Gln
         35                  40                  45

gct gct ttc tgc ttc tca gag acc atc ccg gcc ccc aca ggc aag gag  192
Ala Ala Phe Cys Phe Ser Glu Thr Ile Pro Ala Pro Thr Gly Lys Glu
     50                  55                  60

gag gcc cag cag aga acc gac atg gaa ttg ctt cgc ttc tcg ctg ctg  240
Glu Ala Gln Gln Arg Thr Asp Met Glu Leu Leu Arg Phe Ser Leu Leu
 65                  70                  75                  80

ctc atc cag tca tgg ctg ggg ccc gtg cag ttc ctc agc agg att ttc  288
Leu Ile Gln Ser Trp Leu Gly Pro Val Gln Phe Leu Ser Arg Ile Phe
                 85                  90                  95

acc aac agc ctg atg ttc ggc acc tcg gac cgt gtc tat gag aaa ctg  336
Thr Asn Ser Leu Met Phe Gly Thr Ser Asp Arg Val Tyr Glu Lys Leu
            100                 105                 110

aag gac ctg gaa gag ggc atc cag gct ctg atg cag gag ctg gaa gat  384
Lys Asp Leu Glu Glu Gly Ile Gln Ala Leu Met Gln Glu Leu Glu Asp
        115                 120                 125

ggc agc ccc cgt gtt ggg cag atc ctc aag caa acc tat gac aag ttt  432
Gly Ser Pro Arg Val Gly Gln Ile Leu Lys Gln Thr Tyr Asp Lys Phe
    130                 135                 140

gac gcc aac atg cgc agc gac gac gcg ctg ctc aaa aac tat ggg ctg  480
Asp Ala Asn Met Arg Ser Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu
145                 150                 155                 160

ctc tcc tgc ttc aag aag gac ctg cac aaa gcg gag acc tac ctg cgg  528
Leu Ser Cys Phe Lys Lys Asp Leu His Lys Ala Glu Thr Tyr Leu Arg
                165                 170                 175

gtc atg aag tgt cgc cgc ttt gtg gaa agc agc tgt gcc ttc tag      573
Val Met Lys Cys Arg Arg Phe Val Glu Ser Ser Cys Ala Phe
            180                 185                 190
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<210> 43
<211> 190
<212> PRT
<213> Mus musculus

<400> 43
Phe Pro Ala Met Pro Leu Ser Ser Leu Phe Ser Asn Ala Val Leu Arg
1 5 10 15

Ala Gln His Leu His Gln Leu Ala Ala Asp Thr Tyr Lys Glu Phe Glu
20 25 30

Arg Ala Tyr Ile Pro Glu Gly Gln Arg Tyr Ser Ile Gln Asn Ala Gln
35 40 45

Ala Ala Phe Cys Phe Ser Glu Thr Ile Pro Ala Pro Thr Gly Lys Glu
50 55 60

Glu Ala Gln Gln Arg Thr Asp Met Glu Leu Leu Arg Phe Ser Leu Leu
65 70 75 80

Leu Ile Gln Ser Trp Leu Gly Pro Val Gln Phe Leu Ser Arg Ile Phe
85 90 95

Thr Asn Ser Leu Met Phe Gly Thr Ser Asp Arg Val Tyr Glu Lys Leu
100 105 110

Lys Asp Leu Glu Glu Gly Ile Gln Ala Leu Met Gln Glu Leu Glu Asp
115 120 125

Gly Ser Pro Arg Val Gly Gln Ile Leu Lys Gln Thr Tyr Asp Lys Phe
130 135 140

Asp Ala Asn Met Arg Ser Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu
145 150 155 160

Leu Ser Cys Phe Lys Lys Asp Leu His Lys Ala Glu Thr Tyr Leu Arg
165 170 175

Val Met Lys Cys Arg Arg Phe Val Glu Ser Ser Cys Ala Phe
180 185 190

<210> 44
<211> 573
<212> DNA
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20 25 30

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Arg Ala Tyr Ile Pro Glu Gly Gln Arg Tyr Ser Ile Gln Asn Ala Gln
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gct gct ttc tgc ttc tca gag acc atc ccg gcc ccc aca ggc aag gag   192
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ctc atc cag tca tgg ctg ggg ccc gtg cag ttc ctc agc agg att ttc   288
Leu Ile Gln Ser Trp Leu Gly Pro Val Gln Phe Leu Ser Arg Ile Phe
                 85                  90                  95

acc aac agc ctg atg ttc ggc acc tcg gac cgt gtc tat gag aaa ctg   336
Thr Asn Ser Leu Met Phe Gly Thr Ser Asp Arg Val Tyr Glu Lys Leu
            100                 105                 110

aag gac ctg gaa gag ggc atc cag gct ctg atg cag gag ctg gaa gat   384
Lys Asp Leu Glu Glu Gly Ile Gln Ala Leu Met Gln Glu Leu Glu Asp
        115                 120                 125

ggc agc ccc cgt gtt ggg cag atc ctc aag caa acc tat gac aag ttt   432
Gly Ser Pro Arg Val Gly Gln Ile Leu Lys Gln Thr Tyr Asp Lys Phe
    130                 135                 140

gac gcc aac atg cgc agc gac gac gcg ctg ctc aaa aac tat ggg ctg   480
Asp Ala Asn Met Arg Ser Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu
145                 150                 155                 160

ctc tcc tgc ttc aag aag ggc ctg cac aaa gcg gag acc tac ctg cgg   528
Leu Ser Cys Phe Lys Lys Gly Leu His Lys Ala Glu Thr Tyr Leu Arg
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<400> 45

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Arg Ala Tyr Ile Pro Glu Gly Gln Arg Tyr Ser Ile Gln Asn Ala Gln
         35                  40                  45

Ala Ala Phe Cys Phe Ser Glu Thr Ile Pro Ala Pro Thr Gly Lys Glu
     50                  55                  60

Glu Ala Gln Gln Arg Thr Asp Met Glu Leu Leu Arg Phe Ser Leu Leu
 65                  70                  75                  80

Leu Ile Gln Ser Trp Leu Gly Pro Val Gln Phe Leu Ser Arg Ile Phe
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85 90 95

Thr Asn Ser Leu Met Phe Gly Thr Ser Asp Arg Val Tyr Glu Lys Leu
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Asp Ala Asn Met Arg Ser Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu
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gaa gcc tat atc cca aag gaa cag aag tat tca ttc ctg cag aac ccc  144
Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro
         35                  40                  45

cag acc tcc ctc tgt ttc tca gag tct att ccg aca ccc tcc aac agg  192
Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg
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gag gaa aca caa cag aaa tcc aac cta gag ctg ctc cgc atc tcc ctg  240
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ctg ctc atc cag tcg tgg ctg gag ccc gtg cag ttc ctc agg agt gtc  288
Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
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ttc gcc aac agc ctg gtg tac ggc gcc tct gac agc aac gtc tat gac  336
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ctc cta aag gac cta gag gaa ggc atc caa acg ctg atg ggg agg ctg  384
Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu
        115                 120                 125

gaa gat ggc agc ccc cgg act ggg cag atc ttc aag cag acc tac agc  432
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    130                 135                 140

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145                 150                 155                 160

ggg ctg ctc tac tgc ttc agg aag ggc atg gac aag gtc gag aca ttc  528
Gly Leu Leu Tyr Cys Phe Arg Lys Gly Met Asp Lys Val Glu Thr Phe
                165                 170                 175

ctg cgc atc gtg cag tgc cgc tct gtg gag ggc agc tgt ggc ttc tag  576
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Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu
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Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
                 85                  90                  95

Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp
            100                 105                 110

Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu
        115                 120                 125

Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser
    130                 135                 140

Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr
145                 150                 155                 160

Gly Leu Leu Tyr Cys Phe Arg Lys Gly Met Asp Lys Val Glu Thr Phe
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gcc cat cgt ctg cac cag ctg gcc ttt gac acc tac cag gag ttt gaa   96
Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu
             20                  25                  30

gaa gcc tat atc cca aag gaa cag aag tat tca ttc ctg cag aac ccc   144
Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro
         35                  40                  45

cag acc tcc ctc tgt ttc tca gag tct att ccg aca ccc tcc aac agg   192
Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg
     50                  55                  60

gag gaa aca caa cag aaa tcc aac cta gag ctg ctc cgc atc tcc ctg   240
Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu
 65                  70                  75                  80

ctg ctc atc cag tcg tgg ctg gag ccc gtg cag ttc ctc agg agt gtc   288
Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
                 85                  90                  95
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ttc gcc aac agc ctg gtg tac ggc gcc tct gac agc aac gtc tat gac   336
Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp
            100                 105                 110

ctc cta aag gac cta gag gaa ggc atc caa acg ctg atg ggg agg ctg   384
Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu
        115                 120                 125

gaa gat ggc agc ccc cgg act ggg cag atc ttc aag cag acc tac agc   432
Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser
    130                 135                 140

aag ttc gac aca aac tca cac aac gat gac gca cta ctc aag aac tac   480
Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr
145                 150                 155                 160

ggg ctg ctc tac tgc ttc agg aag gac atg gac aag gtc gag aca ttc   528
Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe
                165                 170                 175

ctg cgc atc gtg cag tgc cgc tct gtg gag ggc agc tgt ggc ttc tag   576
Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
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             20                  25                  30

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         35                  40                  45

Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg
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Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu
 65                  70                  75                  80

Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
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Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu
        115                 120                 125

Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser
    130                 135                 140

Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr
145                 150                 155                 160

Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe
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                    165                 170                 175

Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
            180                 185                 190
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(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2001 (04.10.2001)

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(10) International Publication Number
WO 01/72119 A3

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(21) International Application Number: PCT/EP01/03733

(22) International Filing Date: 2 April 2001 (02.04.2001)

(25) Filing Language: English

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(30) Priority Data:
100 16 083.2 31 March 2000 (31.03.2000) DE

(71) Applicant *(for all designated States except US)*: **INGENIUM PHARMACEUTICALS AG** [DE/DE]; Fraunhoferstrasse 13, 82152 Martinsried (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CORNALI, Emmanuelle** [FR/DE]; Herzogstrasse 84, 80796 München (DE). **NEHLS, Michael, Christian** [DE/DE]; Paul-Keller-Strasse 6, 82131 Stockdorf (DE). **SCHLÜTER, Volker** [DE/DE]; Gilmstrasse 64, 81377 München (DE). **WATTLER, Frank** [DE/DE]; Bennostrasse 11a, 82131 Stockdorf (DE). **WATTLER, Sigrid** [DE/DE]; Bennostrasse 11 A, 82131 Stockdorf (DE). **WOLF, Stefan** [DE/DE]; Josef-Ritz-Weg 90, 81673 Münich (DE).

(74) Agents: **VOSSIUS, Volker** et al.; Holbeinstrasse 5, 81679 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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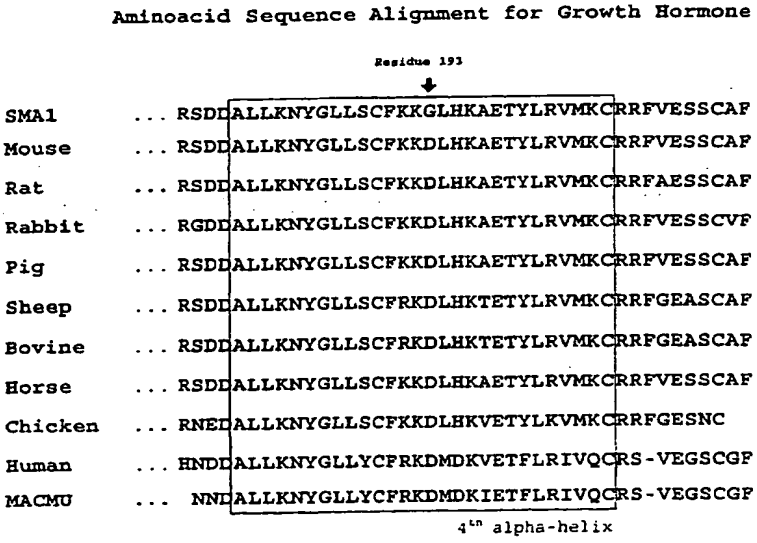
(54) Title: NON-HUMAN ANIMAL MODEL FOR GROWTH DEFICIENCY AND INFORMATION PROCESSING OR COGNITIVE FUNCTION DEFECTS AND USE THEREOF



(57) Abstract: The present invention provides a non-human animal model, particularly a mouse model, for growth deficiency and information procesing or cognitive function defects, in which modified growth hormone is expressed. The modified growth hormone and nucleic acids coding therefor are also provided, as are the correspondingly modified recombinant mouse and human growth hormones. The invention further provides uses for the non-human animal model and the modified growth hormones, in particular for the modified human growth hormone in treating medical conditions associated with over-expression of growth hormone or IGF-1.

WO 01/72119 A3

Published:
— *with international search report*

(88) Date of publication of the international search report:
7 February 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/EP 01/03733

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/61 A01K67/027 C12N15/18 C12N5/10 C07K16/26
A61K38/27 A61K39/395 A61K49/00 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A01K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 04788 A (GENENTECH INC) 3 May 1990 (1990-05-03) claim 38	13,14, 16, 18-23, 26,27, 32-37
A	JIN L ET AL : "High resolution functional analysis of antibody-antigen interactions" J MOL BIOL, vol. 226, no. 3, 5 August 1992 (1992-08-05), pages 851-865, XP001030520 the whole document	13,14, 16, 18-23, 26,27, 32-37

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 2001	05/11/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lonnoy, O

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SMITH L ET AL: "Essential role of growth hormone in ischemia-induced retinal neovascularization" SCIENCE, vol. 276, no. 5319, 13 June 1997 (1997-06-13), pages 1706-1709, XP002179757 cited in the application the whole document	1-44
A	WO 99 46298 A (KERKHOF PETRUS JOHANNES MARIA ;UNIV UTRECHT (NL); GOVERS ROLAND MA) 16 September 1999 (1999-09-16)	
A	WICAR S ET AL: "Conformational changes in the reversed phase liquid chromatography of recombinant human growth hormone as a function of organic solvent: the molten globule state" ANAL CHEM, vol. 66, no. 22, 15 November 1994 (1994-11-15), pages 3908-3915, XP002179758	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ...nal Application No
PCT/EP 01/03733

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9004788 A	03-05-1990	AT 189526 T	15-02-2000
		CA 2001774 A1	28-04-1990
		DE 68929151 D1	09-03-2000
		DE 68929151 T2	20-07-2000
		EP 0397834 A1	22-11-1990
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		WO 9946298 A2	16-09-1999